

SERUM RESPONSE FACTOR-DEPENDENT REGULATION OF SMOOTH
MUSCLE GENE TRANSCRIPTION

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ABSTRACT

Meng Chen

SERUM RESPONSE FACTOR-DEPENDENT REGULATION OF SMOOTH MUSCLE GENE TRANSCRIPTION

Several common diseases such as atherosclerosis, post-angioplasty restenosis, and graft vasculopathies, are associated with the changes in the structure and function of smooth muscle cells. During the pathogenesis of these diseases, smooth muscle cells have a marked alteration in the expression of many smooth muscle-specific genes and smooth muscle cells undergo a phenotypic switch from the contractile/differentiated status to the proliferative/dedifferentiated one. Serum response factor (SRF) is the major transcription factor that plays an essential role in coordinating a variety of transcriptional events during this phenotypic change. The first goal of my thesis studies is to determine how SRF regulates the expression of smooth muscle myosin light chain kinase (smMLCK) to mediate changes in contractility. Using a combination of transgenic reporter mouse and knockout mouse models I demonstrated that a CArG element in intron 15 of the *mylk1* gene is necessary for maximal transcription of smMLCK. SRF binding to this CArG element modulates the expression of smMLCK to control smooth muscle contractility. A second goal of my thesis work is to determine how SRF coordinates the activity of chromatin remodeling enzymes to control expression of microRNAs that regulate the phenotypes of smooth muscle

cells. Using both mouse knockout models and *in vitro* studies in cultured smooth muscle cells I showed how SRF acts together with Brg1-containing chromatin remodeling complexes to regulate expression of microRNAs-143, 145, 133a and 133b. Moreover, I found that SRF transcription cofactor myocardin acts together with SRF to regulate expression of microRNAs-143 and 145 but not microRNAs-133a and 133b. SRF can, thus, further modulate gene expression through post-transcriptional mechanisms via changes in microRNA levels. Overall my research demonstrates that through direct interaction with a CArG box in the *mylk1* gene, SRF is important for regulating expression of smMLCK to control smooth muscle contractility. Additionally, SRF is able to harness epigenetic mechanisms to modulate expression of smooth muscle contractile protein genes directly and indirectly via changes in microRNA expression. Together these mechanisms permit SRF to coordinate the complex phenotypic changes that occur in smooth muscle cells.

B. Paul Herring, Ph.D., Chair

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LIST OF ABBREVIATIONS

ACLP	aortic carboxypeptidase-like protein
ADD3	adducin-3
ATP	adenosine triphosphate
BAF	BRG1- or HRBM-associated factors
Barx1b	Barx homeobox 1
Brg1	brahma-related gene 1
Brm	brahma
CArg	CC(A/T-rich)GG
CHD	chromodomain helicase DNA binding protein
ChIP	chromatin immunoprecipitation
Cre	Cre combinase
CT	computed tomography
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DN-Brg1	dominant negative-Brg1
DTT	dithiothreitol
E10.5	embryonic day 10.5
ECL	enhanced chemiluminescence solution
ER	endoplasmic reticulum
ET1	endothelin 1
ETS	E-twenty six
FBS	fetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
Flk1	fetal liver kinase 1
Flp	flippase recombinase
GATA6	GATA binding protein 6
GFP	green fluorescent protein
GI tract	gastrointestinal tract
H3K4dMe	histone H3 Lysine4 di-methylation
HA	hemagglutinin
HBSS	Hanks buffered saline solution
HDAC	histone deacetylase
HeLa cell	Henrietta Lacks cell
HRP	horseradish peroxide
INO80	chromatin-remodeling ATPase INO80
ISWI	Imitation SWI
Klf4	Krüppel-like factor 4
MADS	mcm1, agamous, deficiens, SRF
MAPK	mitogen-activated protein kinase
miR	microRNA
MKL-1	megakaryoblastic leukemia 1
MKL-2	megakaryoblastic leukemia 2

MLCK	smooth muscle myosin light chain kinases
MRTFA	myocardin-related transcription factor A
MRTFB	myocardin-related transcription factor B
Nkx3.2	Nk3 homeobox 2
PBS	phosphate buffered saline
PDGF-BB	platelet-derived growth factor-BB
PIAS	protein inhibitor of activated STAT
PSS	physiological saline solution
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real-time polymerase chain reaction
RAP74	RNA polymerase II-associating protein 74
RIN	RNA integrity number
RIPA	radioimmune precipitation assay
RISC	RNA-induced silencing complex
RLC	regulatory light chain
RNA	ribonucleic acid
SAF-A	nuclear scaffold attachment factors A
SAF-B	nuclear scaffold attachment factors B
SAP family	SAF-A and B, Acinus, PIAS
S100 β	S calcium binding protein beta
SM22 α	smooth muscle protein 22 alpha
SM α -actin	smooth muscle alpha-actin
SMC	smooth muscle cell
SM MHC	smooth muscle myosin heavy chain
Sox10	SRY (sex determining region Y)-box 10
Sox17	SRY (sex determining region Y)-box 17
SRF	serum response factor
Srgap1	slit-Robo GTPase-activating protein 1
Srgap2	slit-Robo GTPase-activating protein 2
Ssh2	sling-shot 2 phosphatase
STAT	signal transducer and activator of transcription
SWI/SNF	SWItch/Sucrose NonFermentable
TCA	trichloroacetic acid
TCF	ternary complex factor
TFIIF	transcription factor IIF
TGF β	transforming growth facto β
UTR	un-translated region
YFP	yellow fluorescent protein

CHAPTER I

INTRODUCTION

A. Overview of smooth muscle

Smooth muscle is traditionally classified as either multi-unit or single-unit. In multi-unit smooth muscles, each muscle fiber operates independent of each other and usually is innervated by a single nerve ending. Examples of this type of smooth muscle include the ciliary muscle of the eye, the iris of the eye and the piloerector muscle that causes erection of hair. In single-unit smooth muscle, a whole group of smooth muscle cells function together as a single unit. The cells are aggregated into bundles and their cell membranes are adherent to each other. In addition, their cell membranes are joined by many gap junctions through which ions can pass from one cell to another to facilitate coordinated contraction of a group of smooth muscle fibers. Because this type of smooth muscle occupies the walls of most viscera of the body, such as gastrointestinal tract, bile duct, genitourinary tract, uterus, bladder and some blood vessels, they are also often called visceral smooth muscle [1].

Unlike skeletal and cardiac muscle, smooth muscle is not striated. In striated muscles, actin and myosin filaments are highly organized into sarcomeres, that give rise to the alternating light bands that contain only actin filaments, and dark bands that consist of both myosin filaments and overlapping actin filaments

(Figure 1). However, in smooth muscles, although there are both actin and myosin filaments with similar but not exactly the same properties as those in striated muscle, they are not organized into sarcomeres. Instead, the actin filaments are attached to dense bodies that are either attached to the cell membrane or dispersed inside the cell, while the myosin filaments are interspersed among the many actin filaments, lacking the regularity of striated muscle, as shown diagrammatically in Figure 1.

B. Smooth muscle contraction

Similar to striated muscles, smooth muscle cells (SMCs) contract in response to the movement of actin and myosin filaments that is stimulated by a rise in intracellular calcium ions. However, the way that calcium ions stimulate the movement of actin and myosin filaments is distinct between striated and smooth muscle tissues. In striated muscles, calcium ions act via the troponin-tropomyosin complex to trigger contraction. In contrast, smooth muscle cells do not have troponin but rather have a high concentration of calmodulin, another calcium binding protein. In smooth muscle, when levels of intracellular calcium ions rise, they bind to calmodulin and the calcium-calmodulin complex then activates myosin light chain kinase (MLCK), which phosphorylates serine 19 of the 20kDa regulatory light chain of myosin. Phosphorylation of myosin light chain stimulates the actin-activated ATPase activity of myosin, thereby starting the attachment-detachment crossbridge cycle and triggering the contraction of

smooth muscle cells. During relaxation, a decrease in intracellular calcium ion concentration results in attenuation of MLCK activity and myosin light chain phosphatase de-phosphorylates the myosin light chains (Figure 2). As MLCK is crucial for controlling smooth muscle contractility, alterations in MLCK expression are associated with diseases that affect this process. For example, alterations in MLCK expression are involved in a variety of pathologies, including colitis [2], inflammatory bowel disease [3], asthma [4,5], inflammatory lung disease [6], familial aortic dissection [7] and hypertension [8,9]. Because of the importance of MLCK expression levels in physiological and pathological processes, it is important to understand the transcriptional mechanisms that govern MLCK expression. Experiments described in Chapter II will identify the key transcriptional pathways that regulate MLCK expression *in vivo* in mice to control smooth muscle contractility.

C. Smooth muscle origins

Smooth muscle cells in different tissues have very diverse embryonic origins. It is generally accepted that smooth muscle cells in most abdominal organs are derived from local mesoderm or mesothelium during embryogenesis [10]. The origins of vascular smooth muscle cells are particularly heterogenous [11]. Lelievre and Le Douarin revealed that smooth muscle cells of aortic arch, ductus arteriosus, and pulmonary artery originate from neural crest through their pioneering studies on chimeric quail-chick embryos. More specifically, they

showed it is the cranial neural crest rather than trunk neural crest or mesencephalic neural crest, that is competent to form smooth muscle cells in these specific vascular regions [12]. More recently, analysis of transgenic reporter mice has suggested that smooth muscle cells in the descending aorta are derived from somites, not lateral plate mesoderm [13]. In contrast, coronary smooth muscle cells have been shown to derive from the proepicardium, which is a transient villous structure formed from the splanchnopleural mesoderm of the posterior heart field. Through cellular lineage tracing, Mikawa and Gourdie reported that the proepicardium contains a population of coronary smooth muscle progenitor cells that migrate into the heart. In addition to coronary smooth muscle cells, data from lineage tracing showed that proepicardial cells also give rise to epicardium, coronary endothelium and cardiac fibroblasts [14,15]. Furthermore, several studies have suggested that some vascular smooth muscle cells arise from endothelial cells or endothelial progenitor cells. For example, DeRuiter *et al.* reported that quail embryonic endothelial cells labeled by wheat germ agglutinin-gold particles relocate to subendothelial layer after 19 hours of incubation and express smooth muscle marker smooth muscle alpha-actin [16]. Yamashita *et al.* demonstrated that the Flk1-positive embryonic stem cells are a type of vascular progenitor cell and they can give rise to both endothelial cells and mural cells, forming vascular structure in chick embryo [17].

D. Smooth muscle phenotypes

In contrast to skeletal muscle and cardiac myocytes that are terminally differentiated, smooth muscle cells are highly plastic and exhibit a spectrum of phenotypic states ranging from robustly synthetic/proliferative smooth muscle cells to the highly contractile/fully differentiated mature cells. In normal adult tissues, smooth muscle cells are quiescent and express high levels of contractile proteins permitting them to control tissue contractility. During the pathogenesis of atherosclerosis, post-angioplasty restenosis, and graft vasculopathies, smooth muscle cells undergo a phenotypic switch from the contractile state to a dedifferentiated, proliferative state in response to a variety of stimuli. The terminology of smooth muscle cell phenotypic modulation was first introduced in 1979, by Campbell *et al.*, based on the observation that a majority of mitotic smooth muscle cells contain reduced myofilament bundles and increased cytoplasmic organelles such as free ribosomes and rough endoplasmic reticulum (ER). Thus, they proposed that modulation of the differentiated phenotype is a prerequisite for smooth muscle cell proliferation [18]. Although the initial identification of smooth muscle cell phenotype depended largely on morphological criteria, over the past several decades, its definition has expanded to encompass alterations of structure, function and gene expression. Fully differentiated contractile smooth muscle cells are spindle-shaped, rich in myofilaments in the cytosol and nonproliferative with low levels of migration, while smooth muscle cells of a dedifferentiated proliferative phenotype are characterized with fewer myofilament bundles and being active in synthesis, secretion, migration as well as mitosis.

Although smooth muscle cells switch from differentiated to proliferative phenotype during the pathogenesis of many diseases, smooth muscle differentiation and proliferation are not necessarily mutually exclusive processes. For example, mouse embryonic smooth muscle cells, before embryonic day 18-20, exhibit very fast replication (75%-80% per day) and have the ability to proliferate under serum-deprived conditions [19], yet at this time the smooth muscle cells are also actively inducing smooth muscle differentiation gene expression [20]. Conversely, SMCs within advanced atherosclerotic lesions show low rates of proliferation, yet have reduced expression of smooth muscle differentiation markers [21]. Therefore, the cessation of proliferation alone is not sufficient to promote smooth muscle differentiation and there must be other additional factors involved in initiating smooth muscle differentiation [22].

In adults, it is traditionally accepted that dedifferentiated proliferating smooth muscle cells that are seen under pathological conditions have arisen from the previously fully differentiated mature smooth muscle cells. However, there are also reports that these pathological smooth muscle cells may arise from a variety of stem cells and smooth muscle progenitor cells. Several studies have shown that bone marrow derived cells, probably hematopoietic stem cells or circulating smooth muscle progenitor cells, can contribute to the dedifferentiated smooth muscle cell population in the neointima following vascular injury [23,24,25,26]. Recently, a new type of progenitor cell, named a multipotent vascular stem cell,

which expresses markers such as Sox10, Sox17 and S100 β , has been identified in the vascular walls [27]. In response to vascular injury, these multipotent vascular stem cells, not pre-existing differentiated smooth muscle cells, were shown to proliferate and contribute to the neointima formation [27]. Since this conclusion challenges the long-standing paradigm that the dedifferentiation of smooth muscle cells from contractile to proliferative status plays an important role in vascular remodeling, it has evoked an intense debate on this controversial topic among a group of leaders in the smooth muscle pathology field [28,29].

E. Smooth muscle differentiation markers

To better discriminate the different states of smooth muscle cells, many smooth muscle cell-specific differentiation markers have been investigated. This analysis has revealed that most smooth muscle differentiation markers are not exclusively specific to smooth muscle cells. Instead they are transiently expressed in other cell types. Reported smooth muscle selective markers include: smooth muscle alpha-actin (SM α -actin), SM22 α , telokin, smooth muscle myosin heavy chain (SM MHC), calponin, aortic carboxypeptidase-like protein (ACLP), desmin, h-caldesmon, meta-vinculin, and smoothelin [22,30,31]. Some of these smooth muscle markers are discussed in more detail below.

SM α -actin is the most widely used smooth muscle marker and fortunately there are several highly selective antibodies for this protein available commercially. It is

expressed in smooth muscle at a very early stage and it is the first specific protein known to be expressed during smooth muscle cell development [32]. In addition, it is the most abundant protein in differentiated smooth muscle cells comprising up to 40% of total cellular protein [33]. However, it is not specifically expressed in smooth muscle cells and is expressed in a variety of non-smooth muscle cells under certain conditions, such as striated muscle cells during development [34], fibroblast cells during wound healing [35], endothelial cells during vascular remodeling and its expression remains high in dedifferentiated smooth muscle cells [36].

SM22 α is a 22kDa smooth muscle cell-restricted protein that physically associates with actin filaments in contractile smooth muscle cells although its physiological function is unclear. A gene knockout study suggested it is not required for the basal functions mediated by vascular or visceral SMCs during mouse development [37]. Although SM22 α is restricted to smooth muscle cells in adult tissues, it is transiently expressed in skeletal muscle and cardiac muscle during embryogenesis [37,38]. Compared with smooth muscle α -actin, SM22 α starts to appear a little later around embryonic day 9.5 during embryonic development [39].

Telokin is a 17kDa protein transcribed from an independent intronic promoter within the *mylk1* gene. Telokin plays a role in calcium de-sensitization of smooth muscle contraction [40,41,42]. It is a very specific marker for smooth muscle

differentiation, although its expression in vascular smooth muscle is much lower than that in visceral smooth muscle.

SM MHC is perhaps the most discriminating marker for smooth muscle cell characterization. Using *in situ* hybridization at different mouse embryonic stages, it was shown that SM MHC transcripts begin to appear in early developing aorta at embryonic day 10.5, then in the gut, lung, peripheral blood vessels at day 12.5, and in esophagus, ureter as well as bladder at day 17.5. No SM MHC transcripts were observed in non-smooth muscle tissues during embryogenesis [43]. Consistent with these data, a lacZ reporter driven by the SM MHC promoter plus the first intron was highly specifically expressed in mouse smooth muscle tissues *in vivo* [44]. Furthermore, fate mapping studies using mice with Cre recombinase (Cre) driven by the SM MHC promoter/enhancer crossed with Cre-dependent lacZ reporter mice also demonstrated complete smooth muscle cell specificity, except for a small population of cells within the right atrium at embryonic day 8.5. These cells may represent transiently differentiated SMCs in the process of epithelial-mesenchymal transformation from proepicardial cells into coronary smooth muscle cells [45]. Overall these data suggest that currently SM MHC is the most specific marker of the smooth muscle cell lineage.

F. Transcription factors/cofactors and SMC genes

In order to understand how numerous stimuli affect the phenotypes of smooth muscle cells during SMC pathogenesis, it is necessary to study the transcriptional pathways that drive expression of smooth muscle differentiation markers. Tremendous progress has been made in this area as summarized in Figure 3 [46]. Among all the transcription factors known to regulate smooth muscle cell differentiation, serum response factor (SRF) is perhaps the most central and best studied one.

i. SRF and the SRF response element

SRF was named based on its binding activity to a serum response element whose core sequence is CC(A/T)₆GG and is required for the expression of the immediate early response gene, c-fos, after serum stimulation. In 1985, Treisman found that a 5' proximal segment of the human c-fos promoter was required for its rapid upregulation following growth factor stimulation [47,48]. Further studies identified a 22-bp element (AGATGT CCATATTAGG ACATCT) having inverted repeat of dyad symmetry within that region. Since the element binds to a protein in HeLa cell nuclear extracts harvested after serum stimulation, it was named serum response element [47,49,50]. Meanwhile, Minty and Kedes performed a comparative promoter analysis of human, rat, mouse and chicken cardiac alpha-actin genes and found a strikingly conserved common element CC(A/T-rich)GG named CCArGG box [51]. The CCArGG box (abbreviated as CArG box) is the core sequence of the serum response element. Although the terms serum

response element and CArG box are sometimes used interchangeably, the serum response element contains not only a CArG box but also a binding site for ETS (E-twenty six) transcription factors [52]. As described later, it is in fact the ETS transcription factors that are the direct sensors of growth factor signaling cascades activated by mitogen-activated protein kinase (MAPK).

To predict the location of all possible CArG boxes within the human genome, the Miano group performed a genome-wide screen in an attempt to define the functional mammalian “CArGome”. Comparative genomic analysis between human and mouse, uncovered more than 100 hypothetical genes that include at least one CArG box within 4kb upstream from their transcription start sites. Out of the 89 genes that were manually selected and cloned, 60 target genes were validated by at least two methods, including luciferase reporter assays, gel shift assays, chromatin immunoprecipitation assays, and changes in mRNA expression following RNAi knockdown of SRF. Remarkably, Gene Ontology categorization of CArG element-containing genes revealed that almost half of the validated genes are associated with the actin cytoskeleton or contractile apparatus [53]. Moreover, analysis of 33 smooth muscle cell-restricted genes (Figure 4) showed that 23 genes have one or more evolutionarily conserved CArG boxes, with a total of 37 CArG boxes usually within 2-3kb of the transcription start sites [54]. This finding probably reflects the requirement for SRF to be relatively close to the RNA polymerase in order to activate transcription. Consistent with this, SRF was shown to interact with the RAP74

subunit of TFIIF in the RNA polymerase II holoenzyme and disruption of the interaction impaired SRF activated transcription [55]. Furthermore, analysis of the frequency of nucleotides in the 37 conserved CArG boxes from the 33 smooth muscle cell-restricted genes, revealed some of the highly conserved characteristics adopted by regulatory CArG boxes in smooth muscle specific genes: (1) The most highly conserved nucleotides are the known contact points for SRF (AT -4/-5 and +4/+5). (2) There appears to be a preference for T at -2 and +1 and A at +2 and +3. (3) Most of the flanking nucleotides are more or less evenly distributed, although there is a bias for T at -8, G at +10, A or C at -9 and -15, C or G at -7, -10 and +13, and G or T at +9 and +11 (Figure 4) [54].

Many of the highly conserved CArG boxes located in the promoter regions of smooth muscle contractile genes have been validated to be important for gene transcription both *in vitro* and *in vivo*. For example, transgenic mice generated using a 310bp telokin promoter fragment that includes one CArG element had smooth muscle-specific reporter gene expression similar to that of the endogenous telokin gene, however, mutation of the CArG box completely abrogated the transgene expression [56].

Some smooth muscle cell-restricted genes have multiple CArG boxes in their promoters that are not of equal importance for gene transcription. For example, the SM α -actin gene harbors two CArG-like elements 5' of the transcription start site and a consensus CArG box in intron1. The two proximal 5' CArG elements

were shown to be important for SMC-specific activity in cultured cell lines *in vitro* [57]. However, transgenic mice harboring the proximal SM α -actin promoter sequences that included both of these elements (up to -2800bp) only directed lacZ transgene expression in cardiac and skeletal muscle, but not in SMCs, suggesting the two 5' CArG elements may not be sufficient for SM α -actin expression in SMC *in vivo*. In contrast, a transgene driven by the promoter region together with the first intron essentially recapitulated the endogenous expression pattern. Within this transgene, mutation of the distal 5' CArG abolished promoter activity, while mutation of the proximal 5' CArG box only had a mild effect. Interestingly, mutation of the intronic CArG box specifically abolished its activity in SMCs without affecting expression in striated muscles, suggesting that the intronic CArG box of the SM α -actin gene may determine its smooth muscle cell-restricted expression [58]. While most SMC-specific genes have one or more CArG boxes in their regulatory regions, some however do not have any evolutionarily conserved CArG elements within 10kb upstream of their transcription start sites. This implies the existence of alternative mechanisms directing the smooth muscle specificity, although these have not yet been described in detail.

SRF is a founding member of the MADS (the name refers to the names of originally identified members: Mcm1 in yeast, Agamous and Deficiens in plants, and SRF in animals) domain-containing family of transcription factors. The amino-terminus (N-terminus) of SRF comprises a basic DNA-binding domain, a

dimerization domain and an interface for protein-protein interactions [59], while the carboxyl-terminus (C-terminus) of SRF contains a strong transactivation domain with phosphorylation sites that signal the recruitment of SRF-associated factors [60]. SRF binds to CArG boxes as homodimer. The primary DNA-binding element is an anti-parallel coiled coil of two amphipathic α -helices that were aligned parallel to the minor groove in the center of CArG sequence. The DNA molecule wraps around the coiled coil allowing the basic amino of the α -helices to fit into the DNA major groove (Figure 5) [61].

SRF is an essential transcription factor for mammalian mesoderm formation. Whole body SRF knockout mice have arrested gastrulation development at about embryonic day 7 and have a complete loss of mesoderm. Activation of SRF-dependent immediate early response genes such as *egr-1*, *c-fos* and early smooth muscle marker SM α -actin were severely impaired in knockout embryos [62]. Several groups have generated tissue-specific SRF knockout mice in order to circumvent the embryonic lethality of the global SRF knockout mice. Heart-specific deletion of SRF using a beta MHC-driven Cre transgene resulted in lethal cardiac defects between E10.5 and E13.5 including abnormally thin myocardium, dilated cardiac chambers, poor trabeculation, and a disorganized interventricular septum [63]. Deletion of SRF in cardiac and smooth muscle cells by SM22-Cre mediated excision, also resulted in embryonic death around E11.5 [64]. Similarly, deletion of SRF using alpha MHC-Cre caused embryonic lethality by E12.5 due to cardiac insufficiency [65]. Furthermore, tamoxifen induced SRF

deletion in the heart of adult mice led to dilated cardiomyopathy and death from heart failure after 10 weeks of treatment [66]. In skeletal muscle, knockout of SRF using Cre recombinase resulted in perinatal lethality due to dysmyofibrillogenesis in skeletal muscle [67]. Tamoxifen induced deletion of SRF in adult smooth muscle cells caused both vascular and visceral abnormalities. In the vasculature there was decreased contractile response, reduced smooth muscle contractile proteins and abnormal vascular tone [68]. In the GI tract SRF knockout resulted in impaired intestinal contractility, defective intestinal peristalsis and decreased SMC contractile protein expression [69,70]. Overall these data strongly demonstrated that SRF is an absolutely critical transcription factor for muscle differentiation.

Although SRF is expressed at high levels in smooth muscle tissues, SRF is a ubiquitous transcription factor. Genes mediating two largely opposite processes-proliferation and differentiation, can both possess CArG boxes that are regulated by SRF. Hence expression of SRF alone cannot explain how it orchestrates cell-specific programs of gene expression. It has been reported that several mechanisms may be involved in regulating SRF activity in cell context-dependent manner, such as its association with cofactors, posttranscriptional modification of SRF, variations in SRF binding affinity among different CArG boxes, number, position and spacing of CArG boxes, as well as the chromatin conformation of CArG elements [46]. For the purposes of my research I will focus mainly on the

regulation of SRF activity in SMCs through interaction with SRF transcription cofactors and chromatin remodeling enzymes.

ii. SRF transcription cofactors

Among all the SRF transcription cofactors, myocardin is one of the most important for SMC differentiation [71]. Myocardin was first discovered in an attempt to search for cardiac unique genes and its expression is restricted to cardiac and smooth muscles. Through interacting with the MADS domain of SRF, myocardin and SRF-CArG boxes form a ternary complex that transactivates several cardiac and smooth muscle-specific genes, such as calponin, SM MHC, SM α -actin, and SM22 α , however it does not significantly activate growth response genes such as c-fos [71,72]. Myocardin knockout mice die by embryonic day 10.5 with no evidence of aortic smooth muscle cell differentiation, indicating that myocardin is required for vascular smooth muscle differentiation [73]. Myocardin is thus a tissue-restricted transcription cofactor that helps to explain SRF's ability to direct SMC-restricted gene expression.

Two myocardin related transcription factors, MRTFA/megakaryoblastic leukemia 1 (MKL-1) and MRTFB/MKL-2, have been identified to have similar domain structure as well as transcriptional activity to myocardin [74,75]. Myocardin and MRTFs all belong to the SAP domain family, which is named for SAF-A and B (nuclear scaffold attachment factors A and B), Acinus, a caspase-3-activated

protein required for apoptotic chromatin condensation, and PIAS, a protein inhibitor of activated STAT (signal transducer and activator of transcription) [76]. As schematically depicted in Figure 6, the N termini of myocardin family members contain conserved domains named RPEL domains. However, the diverged RPEL domains in myocardin as compared to the MRTFs permit myocardin to be localized exclusively in the nucleus. In contrast, MRTFA and MRTFB bind to actin through their RPEL motifs, resulting in their cytoplasmic localization under unstimulated conditions [77]. Myocardin binding to SRF is mediated through the B1 domain located between the conserved basic and glutamine-rich domains of myocardin. The SAP domain is highly conserved among myocardin family members, although its physiological function is not clear. The C-terminus of myocardin family members contains a very strong transcriptional activation domain. Distinct from the cardiac and smooth muscle cell-restricted expression pattern of myocardin, MRTFs are ubiquitously distributed. Although MRTFs are not specifically localized to SMCs, both MRTFA and MRTFB play important roles in SMC differentiation. MRTFA-null mice have a defect in myoepithelial cell differentiation with attenuated SMC contractile proteins such as SM α -actin, SM MHC, calponin and tropomyosin [78,79]. Additionally, MRTFB knockout mice die during embryonic development with a spectrum of cardiac outflow defects [80,81]. Mechanistically, it has been reported that regulation of MRTFs shuttling between cytoplasm and nucleus is critical for the smooth muscle-specific effects by these molecules. For example, activation of Rho signaling promotes the assembly of F-actin from monomeric G-actin, thus

the lower concentration of free G-actin in the cytosol increases the accumulation of MRTFs to nucleus, where MRTFs associate with SRF and activate transcription of SMC differentiation genes [77].

iii. Regulation through combination of transcription factors/cofactors

The MADS domain of SRF is a docking site for many other transcription cofactors, such as homeodomain proteins Barx1b [82] and Nkx3.2 [83], the zinc finger protein GATA6 [83]. Although each of these proteins can form a stable ternary complex with SRF-CArG DNA, these cofactors are not SMC restricted, nor can they discriminate CArG boxes within differentiation marker genes from immediate early response genes. However, it has been reported that SRF, Nkx3.2 and GATA6 together cooperatively activate several SMC marker genes, including SM22, but not c-fos [83], implying that combination of several transcription factors or cofactors may provide a mechanism for a general transcription factor such as SRF to control SMC-specific genes selectively.

Numerous signaling pathways converge on the key transcription factor SRF. Constant integration of these many signals present in the local environment, in aggregate, determines the gene expression pattern as well as the phenotypic status of smooth muscle cells, as summarized in Figure 7 [22]. Although relatively little is known about the factors regulating smooth muscle cell phenotypes *in vivo*, many studies in cultured SMCs suggested that a great

variety of factors, including mechanical forces [84], extracellular matrix components such as collagen [85,86,87], endothelial-SMC interaction [88], neuronal factors [89], reactive oxygen species [90], contractile agonists such as vasopressin and angiotensin II [91,92], TGF [93,94], and platelet-derived growth factor-BB (PDGF-BB) [95,96] are able to modulate the expression of SMC differentiation markers in the cultured cell systems. As a coordinator of numerous transcriptional events, SRF is able to integrate different signals influencing SMC phenotypes through its ability to form unique complexes with other transcription cofactors. For example, PDGF stimulation of smooth muscle cells activates a MAPK signaling cascade, leading to phosphorylation of Elk1, a ternary complex factor (TCF) of the ETS-domain family. Phosphorylated Elk1 then forms stable ternary complex with SRF on the ETS binding site located adjacent to CArG boxes, replacing myocardin from the CArG boxes of SMC genes, thus repressing SMC differentiation genes expression [97,98]. At the same time, the phosphorylated Elk1/SRF complex also binds to CArG elements in growth-responsive genes such as c-fos, egr1, inducing their transcription and thereby causing SMC proliferation. In addition to increasing phosphorylated Elk1, PDGF stimulates expression of Krüppel-like transcription factor 4 (Klf4), a transcriptional repressor that antagonizes myocardin-induced activation of SMC genes via binding to G/C-rich cis-acting sequence in the transcriptional regulatory regions controlling SMC contractile genes [99,100]. Since most of these data were derived from observations of *in vitro* cell culture models, these conclusions

should be interpreted with caution as *in vitro* smooth muscle cell culture systems might not fully recapitulate the *in vivo* environment.

G. Chromatin remodeling and SMC genes

In eukaryotic cells, DNA is compacted 5000-fold into chromatin and compartmentalized in the nucleus. The basic unit of chromatin is the nucleosome, which is comprised of 146 base pairs of double stranded DNA wrapping around two copies of histones H2A, H2B, H3 and H4. As a result of this complicated packaging, regulation of DNA accessibility is an important first step for the binding of transcription factors/cofactors during gene transcription. Using chromatin immunoprecipitation (ChIP) assays, it was revealed that SRF has a preferential binding to the promoter CArG boxes of SMC genes with enhanced histone H3 or H4 acetylation [101]. Moreover, signals such as vascular injury recruit Klf4-dependent histone deacetylase (HDAC) activity to SMC gene promoters, resulting in loss of SRF binding and promoting the dedifferentiated phenotype. In contrast, in the absence of Klf4, SRF is able to recognize accessible CArG box sequences within the “open” chromatin containing acetylated histone H4, synergizing with myocardin recruited histone H3 Lysine4 di-methylation (H3K4dMe), to promote transcription of SMC genes and SMC differentiation [102,103].

Chromatin conformation changes are generally controlled by at least three mechanisms: DNA methylation, histone modifications and chromatin remodeling by ATP-dependent chromatin remodeling complexes. ATP-dependent chromatin remodeling complexes are comprised of four families in mammals, SWI/SNF (SWItch/Sucrose NonFermentable), INO80 (chromatin-remodeling ATPase INO80), ISWI (Imitation SWI), and CHD (chromodomain helicase DNA binding protein) complexes that are able to regulate the conformation of nucleosomes by utilizing the energy of ATP [104,105]. Of all the ATP-dependent chromatin remodeling complexes, the SWI/SNF complex is the best-characterized one. It consists of 8-11 components, encoded by 20 different genes with possibly a total of 288 predicted assemblies, forming a very stable complex. SWI/SNF complexes contain one of two alternative ATPases, Brahma-related gene 1 (Brg1) or Brahma (Brm). Both of these proteins are ubiquitously expressed in almost all tissues, and have both redundant and distinct functions in regulating gene expression. For instance, Brg1 can slow down growth of tumor cell lines and induce differentiation, whereas Brm has little effect [106]. Brg mutant mice die early in embryonic development due to growth arrest of the inner cell mass and trophoblast [106], while Brm-null mice are slightly larger than normal [107]. Although Brg1 appears to have some functions that cannot be performed by Brm, several groups have shown that in some situations Brm is able to compensate for Brg1, partially or completely. For example, either Brg1 or Brm containing SWI/SNF complexes can support the ability of myocardin or MRTFA to induce expression of smooth muscle-specific genes [108,109]. Although, knockout of

Brg1 alone in smooth muscle resulted in decreased expression of smooth muscle-specific contractile proteins and impaired GI contractility this phenotype was worse in mice also lacking Brm [110]. These data suggest that Brg1-containing SWI/SNF chromatin remodeling complexes and their interactive transcription factors play an essential role in regulating SMC genes expression and smooth muscle phenotypes.

H. MicroRNAs in smooth muscle cells

Recent studies have suggested that altered microRNA expression contributes to SMC phenotypic changes. MicroRNAs are endogenous small (~22nt) non-coding RNA that can target mRNA for degradation or repress mRNA translation by base pairing with the 3'-untranslated region of targeted mRNA. MicroRNA biogenesis is initiated when primary microRNAs are transcribed by RNA polymerase II. Primary microRNAs contain stem-loop structures and are long, ranging in length from a few hundred to thousands of nucleotides. Primary microRNAs are cleaved by RNase III Drosha and its partner DGCR8, into microRNA precursors that are then transported to cytoplasm by Exportin 5. The RNase Dicer recognizes the precursors in the cytoplasm and processes them to mature microRNA duplexes. The mature microRNA which is about 22 nucleotides long, is assembled into an RNA-induced silencing complex (RISC) which directs microRNA binding to the 3'-UTR complimentary sequence of targeted mRNAs, either decreasing mRNA stability, or inhibiting protein translation [111].

Smooth muscle-specific Dicer knockout mice have decreased microRNA expression and exhibit embryonic lethality, associated with extensive internal hemorrhage, dilated thin-walled blood vessels, decreased smooth muscle cell proliferation, diminished expression of smooth muscle contractile proteins, and loss of actin stress fibers [112] [113]. Moreover, tamoxifen-induced deletion of Dicer specifically in smooth muscle cells of adult mice caused a dramatic reduction of smooth muscle contractile markers and blood pressure [114]. These studies reveal critical roles for microRNAs in the development and maintenance of SMCs.

While Dicer-deficient mice reflected the collective functions of numerous miRNAs in smooth muscle cells, several specific microRNAs have been shown to play roles in the regulation of smooth muscle cell proliferation and differentiation. The most highly studied microRNAs in smooth muscle are microRNA-143 (miR-143) and miR-145 [115,116,117,118,119]. The miR-143 and miR-145 are two mature microRNAs that arise from the same primary transcript. Mice lacking both miR-143 and miR-145 are viable and do not display overt abnormalities in smooth muscle differentiation. However, neointima formation in response to vascular injury was profoundly affected in mice lacking these microRNAs [118,119]. Several putative targets of miR-143/145 have been identified. MiR-145 targets multiple transcription factors that are involved in vascular SMC differentiation, such as Klf4, CamKII δ (Ca²⁺/calmodulin-dependent protein kinases II δ) and

myocardin. Interestingly, miR-145 suppresses the levels of Klf4 and CamKII δ , two negative regulators of SMC differentiation but increases the expression of myocardin, a strong positive regulator of differentiation, consistent with miR-145 promoting the contractile phenotype of vascular SMCs [117]. Unlike smooth muscle-specific deletion of Dicer, loss of miR-143/145 is not embryonic lethal indicating that other miRNAs are also involved in SMC differentiation. In addition to miRs-143/145, miR-21 has been reported to promote proliferation and inhibit apoptosis of vascular SMCs by down-regulating phosphatase and tensin homolog (PTEN) and up-regulating B-cell lymphoma 2 [120]. MiR-221 was also found to increase proliferation and reduce the levels of VSMC contractile proteins [121,122]. Recently, miR-133, which is conventionally considered as a cardiac or *in vitro* skeletal muscle specific microRNA, has been shown to inhibit VSMC proliferation and after balloon injury *in vivo*, at least partially through regulating the expression of Sp1 and moesin [123]. Several other miR-133 targets have been identified that are involved in the regulation of cell proliferation and differentiation, such as SRF and cyclin D2 [124,125].

Although microRNAs are now recognized as intriguing regulators of vascular SMC phenotypic modulation, little is known regarding the transcriptional regulation of microRNA in smooth muscle cells. The observation that miR-143/145 and miR-133 were all up-regulated in differentiated and down-regulated in de-differentiated smooth muscle cells suggests that the known transcriptional regulators of smooth muscle differentiation may be also responsible for the

regulation of these microRNAs. In addition, the SWI/SNF chromatin remodeling complex has been shown to be required for miR-133 expression in skeletal muscle together with the transcription factors myogenin and myoD [126,127]. Experiments described in Chapter III of my thesis begin to unravel the genetic and epigenetic mechanisms that regulate the microRNA expression in smooth muscle cells.

I. Rationale

Serum response factor is the major transcription factor that plays an essential role in coordinating a variety of transcriptional events during smooth muscle cell phenotypic changes. SRF forms transcriptional complexes with myocardin or other transcription cofactors and recruits chromatin remodeling enzymes to CArG boxes in the regulatory regions of smooth muscle specific genes, thereby activating their transcription. These functions of SRF are integrated together with the activities of several microRNAs that are involved in regulating SMC gene expression via post-transcriptional mechanisms. Therefore my thesis work mainly focuses on 1) determining the role of CArG elements in regulating expression of smooth muscle myosin light chain kinase, a key protein that regulates SMC contractility and 2) determining the transcriptional and epigenetic mechanisms that regulate microRNA expression in smooth muscle cells.

During these studies I tested two central hypotheses:

1. A CArG element within intron 15 of the *mylk1* gene is required to specifically modulate transcription of the 130kDa smMLCK.
2. SRF and myocardin recruit SWI/SNF remodeling complexes to the promoters of the miRs-143/145 and miR-133 genes to stimulate microRNA expression in differentiated SMCs.

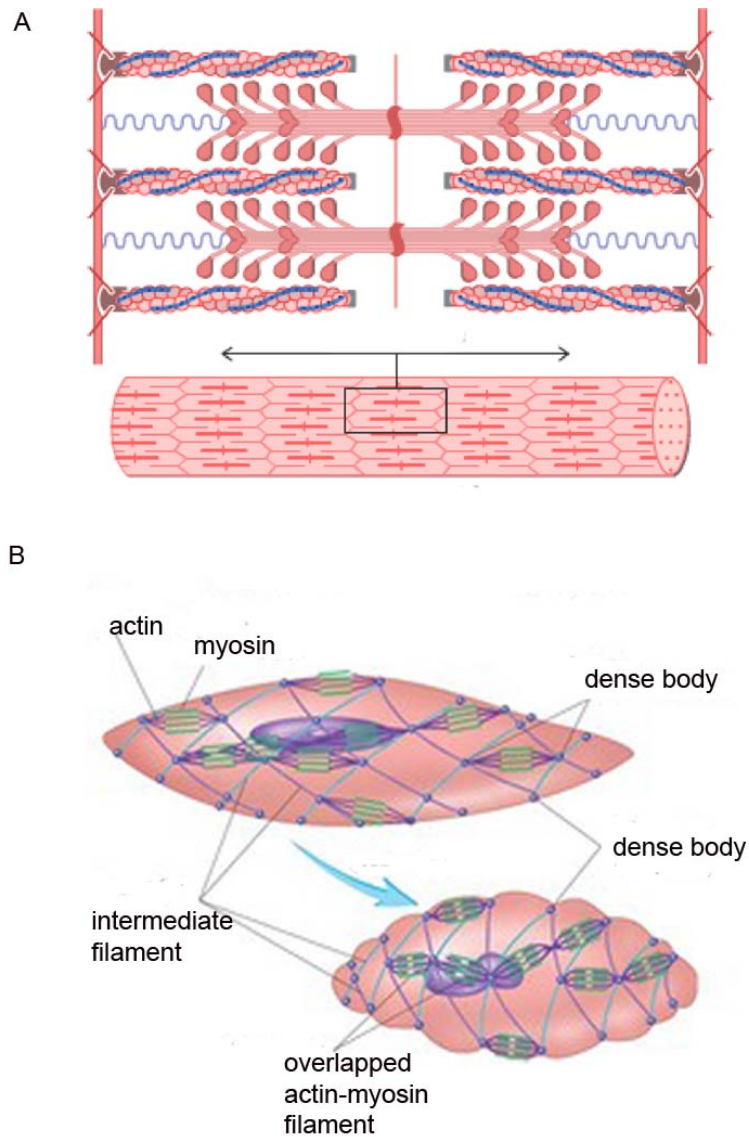


Figure 1. Diagrams show the arrangement of actin and myosin in skeletal and smooth muscle cells.

A. In skeletal muscle cells, actin and myosin filaments are organized into sarcomeres, giving rise to the alternative light bands that contain only actin

filaments, and dark bands that consist of both myosin filaments and the overlapped part of the actin filaments.

B. In smooth muscle cells, the actin filaments are attached to dense bodies that are either attached to the cell membrane or dispersed inside the cell, while the myosin filaments are interspersed among the many actin filaments, lacking the regularity of striated muscle structure.

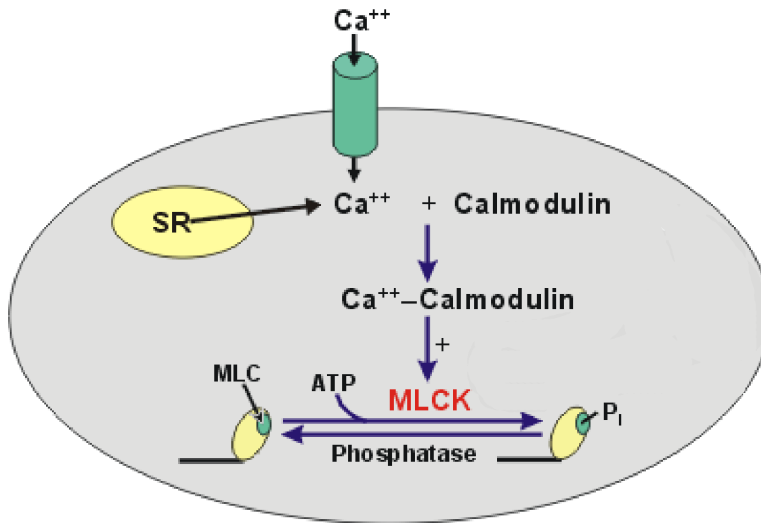


Figure 2. Processes for smooth muscle cell contraction and relaxation

When the intracellular calcium ions concentration is high, calcium ions bind to calmodulin and the calcium-calmodulin complex then activates myosin light chain kinase (MLCK), which phosphorylates the serine 19 of the 20kDa regulatory light chain of myosin. Phosphorylation of myosin light chain allows the myosin head to bind to actin, thereby triggering the contraction of smooth muscle cells. Reversal of the contraction process requires myosin phosphatase that is able to dephosphorylate myosin light chain and detach the actin-myosin binding, thus relaxing smooth muscle cells.

Transcription Factors Known or Likely to be Involved in Regulation of SMC Differentiation/Proliferation	
Transcription Factor	Target Genes
MADS Box Proteins	
SRF MEF2B	Increase numerous SMC differentiation markers Increase SM MHC
Homeodomain Proteins	
Phox1/Mhox Barx1b Barx2b Nkx3.1 Nkx3.2 Hox B7 Gax (Mox2)	Increase SM α -actin Increase β -tropomyosin SMC specific targets unknown Increase SM γ -actin Increase SM22, caldesmon Increase SM22, calponin Increase p21, decrease proliferation
GATA family	
GATA 4/5/6	Increase numerous SMC differentiation markers Increase p21, decrease proliferation
Myocardin	Increase numerous SMC differentiation markers
Zinc Finger Protein	
Sp1/3 Klf4 Klf5	Decrease SM MHC, SM22 Decrease SM22, SM α -actin Increase SM22
Helix-Loop-Helix Protein	
USF Gridlock HAND1 HAND2 Id2/3 Twist	Increase SM α -actin SMC specific targets unknown SMC specific targets unknown SMC specific targets unknown Decrease p21, increase proliferation Decrease SM22
Single-Stranded DNA Binding Proteins	
Pur α , Pur β , MSY1	Decrease SM α -actin
Others	
Egr1	Increase proliferation (numerous targets)

TEF1	Increase SM α -actin
SSRP1	Increase SM22
Mrf2	Increase SM α -actin, SM22
AP1	Increase osteopontin
YY1	Increase SM22
P53	Increase SM α -actin
c-myb	Increase SM α -actin
CRP1/2	SMC specific targets unknown
NFATc1	Increase SM MHC

Figure 3. Transcription factors that are involved in the regulation of smooth muscle cell phenotypic modulation

(Adapted from “Combinatorial control of smooth muscle-specific gene expression” by Kumar, M.S. and G.K. Owens, 2003, Arterioscler Thromb Vasc Biol, 23, p. 737-47)

Gene	Alias	SI ^a	Hsa Chr	Size ^b	Evolutionarily Conserved CArG Boxes (distance from transcription site) ^c
MYH11	SM-MHC	1	16p13.1	155	ccatatttag (-1161), ccttttggg (-1084), ccttttatgg (-965), cctgtatgg (+1584) ccgtttttag (+8132)
MYLKv	Telokin	1	3q21	7.3	ccttttatgg (-66)
SMTNB	Smoothelin B	1	22q12.2	25	cctatttagg (-652)
ACTG2	SM γ -actin	1+	2p13.1	27	gcctataagg (-282), ccttatagg (-110), cctttttagg (-73)
Notch3 *	Notch3	1+	19p13.2	42	None
ITGA8 *	$\alpha 8$ integrin	1+	10p12.32	200	tctttttagg (-4615)
PTK2v *	FRNK	1+	8q24-qter	58	None
CNN1	SM-Calponin	2+	19p13.2	11.5	ctataaatgg (-3120), ccttataagg (+852), ccaagtgtgg (+1530), ccttagtgg (+1745)
APEG1	APEG1	2+	2q36.3	7	None
SMTNA	Smoothelin A	3	22q12.2	15	ccttgaaagg (-934), ccaataagg (-893)
ACTA2	SM α -actin	3+	10q23.3	17	ccctatatgg (-120), cctgtttgg (-70), cctaattagg (+1098)
ITGA1	$\alpha 1$ -integrin	3+	5q11.1	160	ccctttaagg (-156)
EFNB2 *	Ephrin B2	3+	13q33	44	ccagtaaagg (-2078)
TAGLN	SM22a	3+	11q23.2	5.5	ccataaaagg (-273), ccaaatagg (-150)
HEY2 *	CHF-1	3+	6q22.2	13.5	ccaatttagg (-3000)
CSRP1	CRP1	3+	1q32	30	ccatgtatgg (+2800)
MYCD *	Myocardin	4	17p11.2	95	ccaa/ttttg (+6714)
NOV *	CCN3	4	8q24.1	8	None
KCNMB1 *	Maxi-K- β	4	5q34	11	ccatgtttgg (+243)
TPM2	β tropomyosin	4	9p13.2	8	ccctaaaagg (-186)
MRF2a *	MRF2a	4	10q22.1	53	None
BARX2	Barx-2b	4	11q25	75	cccaaaaagg (-167)
MEOX2	Gax	4+	7p22.1	75	None
ELN	Elastin	4+	7q11.23	45	None
DES	Desmin	5	2q35	8	caaataaagg (-3443)
DMD	Dystrophin	5	Xp21.1	2400	cctattatgg (-91)
PGM5 *	Aciculin	5+	9p12-q12	170	None
SRF	SRF	5+	6p21.1	11	ccatataagg (-63), ccataaaagg (-43)
CALD1	h-caldesmon	6	7q33	190	ccaaaaaagg (-309)
VCL	m-vinculin	6	10q23	120	ccttataagg (-262)
ACTN1 *	α -actinin	6	14q24	105	None
AEBP1	ACLP	6	7p15.1	10	None
TPM1	α tropomyosin	6	15q22.1	29	ccaaaaaagt (-87)

Frequency Distribution of Nucleotides Across Flanking and Core CArG Element Sequences in SMC-Restricted Genes

	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10	+11	+12	+13	+14	+15
											C	C	W	W	W	W	W	W	W	G	G									
A	12	6	9	9	9	4	15	4	3	7	0	1	16	12	17	11	25	20	1	0	8	13	15	7	8	6	9	4	7	10
C	11	10	8	16	6	11	10	3	11	10	34	35	4	0	1	0	0	0	0	0	11	10	4	5	9	7	7	11	10	9
G	9	9	12	9	12	12	5	9	14	8	2	0	1	1	5	1	0	2	35	36	8	6	9	13	16	12	8	15	10	12
T	5	12	8	3	10	10	7	21	9	12	1	1	16	24	14	25	12	15	1	1	10	8	9	12	4	12	13	7	10	6
	M	T	G	C	G	S	M	T	S	T	C	C	W	T	A	T	A	A	G	G	C	A	A	K	G	K	T	S	B	G
%	62	32	32	43	32	62	68	57	68	32	92	95	86	65	46	68	68	54	95	97	30	35	41	68	43	65	35	70	81	32

Figure 4. Analysis of the 33 smooth muscle specific genes and the 37 CArG boxes from their regulatory regions

The position of each nucleotide relative to core CArG box is indicated numerically at the top. The numbers in the lower part represent the times and frequency when each indicated base occurs across the 37 CArG sequences.

(Adapted from “Serum response factor: toggling between disparate programs of gene expression” by Miano, J.M., 2003, J Mol Cell Cardiol, 35, p. 577-93)

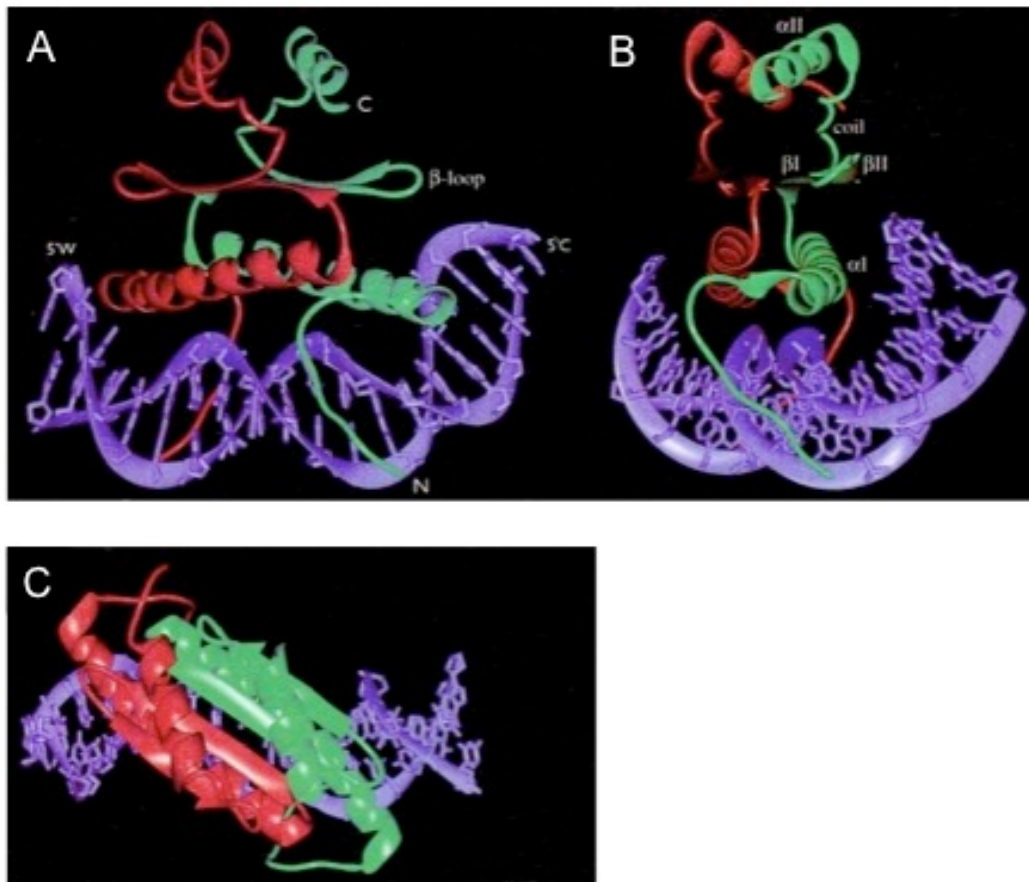


Figure 5. The structure of SRF-DNA binding complex

A. View perpendicular to the molecular two-fold axis and overall DNA helix axis

B. View following the axis of two SRF α -helices, revealing the slab-like structure of SRF monomers

C. View down the molecular two-fold axis

The two SRF monomers, distinguished in red and green, have highly similar conformation; DNA is in purple. The primary DNA-binding element is an anti-parallel coiled coil of two α -helices that were aligned parallel to the minor groove in the center of CArG sequence. The β -sheet packs above the α -helices and is

the central element for homodimerization. The C-terminus contains one short α -helix for each SRF monomer.

(Adapted from “Structure of Serum Response Factor Core Bound to DNA” by Pellegrini, L., T. Song, and T.J. Richmond, 1995, *Nature*, 376: p. 490-498)

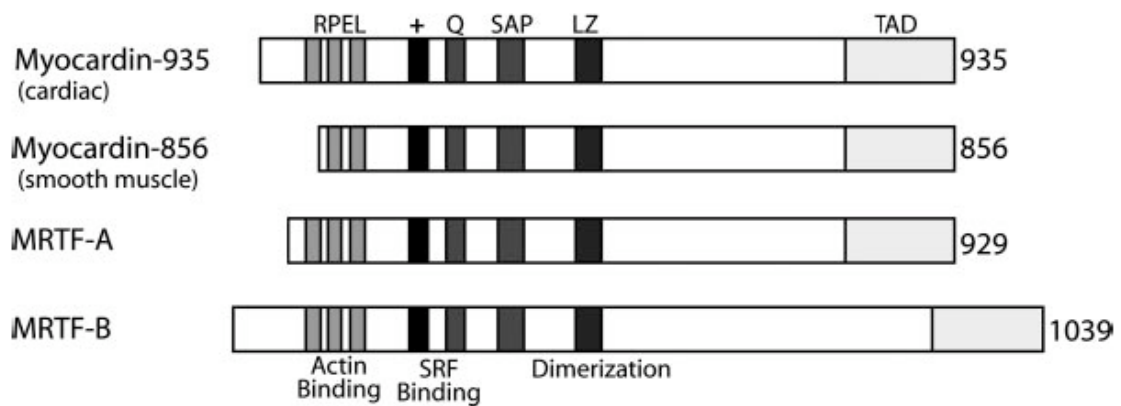


Figure 6. Schematic representation of myocardin and myocardin-related transcription factors

The mouse myocardin gene encodes 935 and 865 amino acid protein isoforms by alternative splicing, with 935-aa isoform predominately in the heart, and 865aa largely in SMCs. The domain structures are designated as labeled in the schematics.

The numbers on the right indicate the number of amino acids for each protein.

The locations of the RPEL, basic (+), glutamine-rich (Q), SAP, LZ, and TAD domains are shown in grey or black rectangles. RPEL domains regulate actin binding in MRTF-A and MRTF-B, yet in myocardin these domains do not bind actin. The basic and glutamine-rich domains are required for SRF binding. LZ domain regulates homo- and heterodimerization with other MRTF family members.

(Adapted from “Actin dynamics control SRF activity by regulation of its coactivator MAL” by Miralles, F., *et al.*, 2003, Cell, 113, p. 329-42)

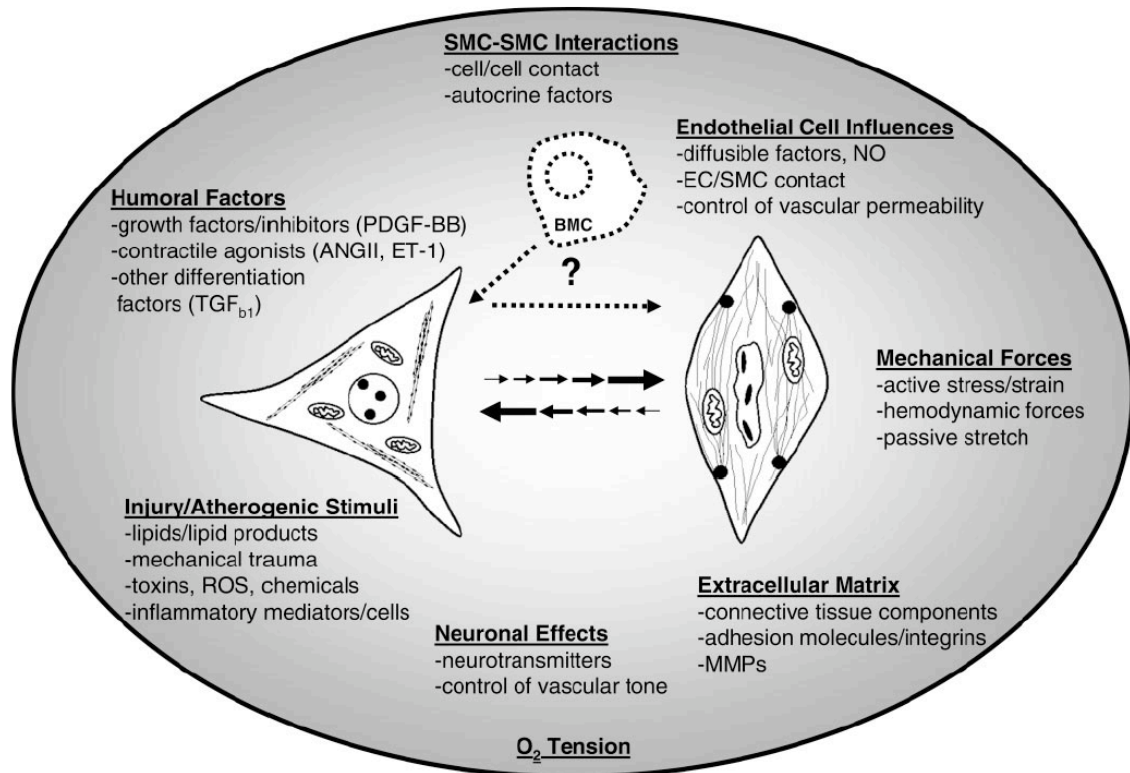


Figure 7. The phenotypic state of smooth muscle cells is highly plastic and dependent on integration of multiple local environmental cues.

This figure summarizes the extrinsic factors that are reported to be important in the regulation of SMC differentiation states. Importantly, smooth muscle cells can exhibit a broad range of phenotypic states depending on the variable expression levels of smooth muscle-specific differentiation markers. The SMC cartoon on left depicts the highly synthetic/proliferative SMC and the one on the right represents the highly contractile fully differentiated/mature SMC.

Two separate pathways are depicted because it is not clear if all the transitions in phenotypes follow the same pathway. The top arrows increase in size depict that the expression levels of smooth muscle differentiation markers such as smooth

muscle alpha-actin and SM MHC increase from left to right, while the lower arrows increase in size from right to left are to show that markers for more immature SMCs are more expressed in the cells on the left. There is controversy as to if bone marrow-derived progenitor cells (BMC, the dashed cell) are capable of becoming fully differentiated SMCs (as indicated by the question mark and the dashed arrows).

PDGF, platelet-derived growth factor; ET, endothelin; TGF, transforming growth factor; ROS, reactive oxygen species; NO, nitric oxide; EC, endothelial cells. (Adapted from “Molecular regulation of vascular smooth muscle cell differentiation in development and disease” by Owens, G.K., M.S. Kumar, and B.R. Wamhoff, 2004, *Physiol Rev*, 84, p. 767-801)

CHAPTER II

REGULATION OF 130KDA SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE EXPRESSION BY AN INTRONIC CARG ELEMENT

A. Summary

The *mylk1* gene encodes a 220kDa nonmuscle myosin light chain kinase (MLCK), a 130kDa smooth muscle MLCK (smMLCK), as well as the non-catalytic product telokin. Together these proteins play critical roles in regulating smooth muscle contractility and changes in their expression are associated with many pathological conditions. It is thus important to understand the mechanisms regulating expression of *mylk1* gene transcripts. Previously we reported a highly conserved CArG element, which binds serum response factor, in intron 15 of *mylk1* [128]. As this CArG element is close to the promoter that drives transcription of the 130kDa smMLCK, we examined its role in regulating expression of the 130kDa smMLCK. Results showed that deletion of the intronic CArG region from a β -galactosidase reporter gene abolished transgene expression in mice *in vivo*. Deletion of the CArG region from the endogenous *mylk1* gene, specifically in smooth muscle cells, decreased expression of the 130kDa smMLCK by 40% without affecting expression of the 220kDa MLCK or telokin. This reduction in 130kDa smMLCK expression resulted in decreased phosphorylation of myosin light chains and attenuated smooth muscle

contractility. The mice with reduced 130kDa smMLCK expression also exhibited a 24% decrease in the length of their small intestines that was associated with a significant reduction of Ki67-positive smooth muscle cells in neonatal mice. Overall these data show that the CArG element in intron 15 of the *mylk1* gene is necessary for maximal expression of the 130kDa smMLCK and that the 130kDa smMLCK isoform is specifically required to regulate smooth muscle contractility and small intestine smooth muscle cell proliferation.

B. Introduction

Myosin light chain kinases (MLCKs) are encoded by four distinct genes, *mylk1-4*. The expression of the *mylk4* gene is poorly characterized, whereas *mylk2* and *mylk3* genes produce MLCK isoforms that are primarily expressed in skeletal muscle and cardiac muscle cells, respectively [129,130]. In contrast, the products of *mylk1* gene are expressed at highest levels in smooth muscle tissues but also detectable in many, if not all, tissues and cell types [131]. The *mylk1* gene is a large gene spanning ~250kb and comprising 31 exons [132]. It encodes at least three protein products: a 220kDa MLCK, a 130kDa MLCK, and a non-catalytic gene product, telokin. Each transcript from the *mylk1* gene is derived from a unique independent promoter within the gene [132]. The 220kDa MLCK is also referred to as nonmuscle MLCK or endothelial MLCK, as it was first characterized in chick embryo fibroblasts and endothelial cells [133,134]. The 130kDa MLCK is also called the smooth muscle MLCK (smMLCK), as it is most abundant in smooth muscle cells, although it is widely expressed in other tissues at lower levels [132,135,136]. Telokin is a non-catalytic product of the gene that is expressed at very high levels in intestinal, urinary, and reproductive tract smooth muscle, at low levels in vascular smooth muscle cells and undetectable levels in other tissues [137].

Both the 220kDa and 130kDa MLCK can phosphorylate serine 19 of the 20kDa myosin regulatory light chain (RLC) of smooth muscle and nonmuscle myosin II,

in the presence of Ca^{2+} and calmodulin. In smooth muscle cells, phosphorylation of the myosin regulatory light chain is an obligatory step for the initiation of contraction. In many other cell types, phosphorylation of RLC induced by MLCK is important for regulating actomyosin-based cytoskeletal functions such as focal adhesion and stress fiber formation, secretion, cytokinesis, neurite growth cone advancement, endothelial and epithelial barrier formation and cell migration [138,139,140,141,142,143,144]. Alterations in MLCK expression have been linked to a variety of pathologies, including colitis [2], inflammatory bowel disease [3], asthma [4,5], inflammatory lung disease [6], familial aortic dissection [7] and hypertension [8,9]. However, the specific functions of the various MLCK isoforms in these processes are not clear. Global knockout of the 220kDa MLCK in mice results in numerous defects in epithelial and endothelial barrier function suggesting that this isoform has a specific role in regulating these processes [145,146,147,148,149]. Through specific targeting of a portion of the catalytic domain shared by the 220 and 130kDa MLCKs, it has been possible to determine the roles of these kinases in specific tissues and cell types [150]. As anticipated, ablation of both MLCK isoforms in smooth muscle cells resulted in impaired contractility and decreased myosin light chain phosphorylation [8,150]. Surprisingly, deletion of both 220kDa and 130kDa MLCK specifically from endothelial cells had very little effect on vascular permeability, bringing into question the importance of endothelial cell expressed MLCK in regulating endothelial barrier function [151]. This surprising finding may suggest that the 220kDa MLCK may be important for regulating endothelial barrier function via

another cell type rather than being important directly only in endothelial cells. Because of the overlapping structure of the 220kDa and 130kDa MLCK it is difficult to specifically examine the function of the 130kDa MLCK without also affecting expression of the 220kDa isoform. To begin to address this issue we examined regulatory elements that specifically regulate expression of the 130kDa MLCK with the idea that deletion of these elements may attenuate expression of the 130kDa MLCK without effecting expression of the other products of the *mylk1* gene. Toward this goal we previously identified a promoter within the *mylk1* gene that specifically directs expression of the 130kDa MLCK [128]. Within this promoter there is a conserved CArG element that binds to serum response factor (SRF) and is required for myocardin-induced expression of the 130kDa MLCK [128].

The CArG element, CC(A/T)₆GG, is the cis-regulatory element that binds serum response factor (SRF), an evolutionarily conserved MADS (MCM1, agamous, deficiens, SRF) domain-containing transcription factor. SRF binding and crystal structure studies have shown that a functional CArG element can deviate by no more than 1 bp from the consensus sequence [152]. Virtually all known CArG elements reside within 4 kb of the transcription start site of genes [152]. Integrating computational algorithm prediction approaches with experimental validation, a genome-wide screen identified 60 target genes that are regulated by CArG elements. Among them, 26 of the validated SRF target genes encode for cytoskeletal/contractile or adhesion proteins [52,152]. When bound to a CArG

element, SRF also provides a docking surface for interaction with numerous accessory co-factors to form ternary complexes, conferring tissue-specific expression of target genes. For example, ternary complexes of SRF and Elk1 are important for growth factors regulation of immediate early genes such as c-fos [153]. In smooth muscle cells, ternary complexes of SRF together with myocardin or myocardin related transcription factors are very powerful activators of numerous smooth muscle-specific contractile and regulatory proteins such as the 130kDa MLCK [72]. In the promoter region of the 130kDa smMLCK (located in intron14 of *mylk1* gene), a highly conserved CArG element has been shown to play a pivotal role in regulating 130kDa smMLCK expression. SRF and its coactivator myocardin enhanced the activity of this promoter and induced expression of the 130kDa smMLCK in 10T1/2 fibroblast cells, while GATA-6 repressed promoter activity, possibly through disrupting SRF-myocardin complexes [128]. Besides the CArG element in the promoter region of the 130kDa MLCK, there is another highly conserved CArG element in the first intron of the 130kDa smMLCK (intron15 of *mylk1* gene). Chromatin immunoprecipitation assays confirmed that this intronic CArG element also binds to SRF in smooth muscle cells [128]. However, the previous studies did not determine if this intronic CArG element effects the expression of the 130kDa smMLCK *in vivo*. In the current study, we found that the intronic CArG element is important for regulating expression of transgenes driven by the 130kDa smMLCK promoter *in vivo* and for driving expression of endogenous 130kDa smMLCK in mice. Moreover, we show that targeting this element is an effective means to

specifically decrease expression of the endogenous 130kDa smMLCK without affecting expression of the 220kDa MLCK or telokin.

C. Methods

i. Generation of targeting vector for homologous recombination

The targeting vector was generated by inGenious Targeting Laboratory (Stony Brook, NY). A 7.56kb fragment containing about 5.1kb extending 5' and 2.16kb extending 3' to the intronic CArG region, was subcloned from a C57BL/6 BAC clone (RP23: 55O1, Source BioScience, Nottingham, UK) into pSP72 vector (Promega, Fitchburg, WI). To construct the targeting vector for homologous recombination, a loxP/FRT flanked Neo cassette was inserted 54bp 3' of the intronic CArG element (smMLCK: +1696-+1705), and a single loxP site containing engineered Afl II and BamH I sites for southern blot analysis was inserted 184bp 5' of the intronic element.

ii. Generation of transgenic reporter mice

The 130kDa smMLCK promoter, exon 1, intron 1 and portion of exon 2 were cut from the pGL2B construct described previously [128] and ligated into the pWhere LacZ reporter vector (InvivoGen, San Diego, CA). SW105 bacteria (NCI-Frederick, MD) that has a flippase recombinase (Flp) inducible gene were transformed with the targeting vector described above and cultured at 32°C. L-arabinose (A3256, Sigma-Aldrich, St. Louis, MO) induced flippase recombinase gene expression was used to excise the Neo cassette from the targeting vector.

The resulting plasmid was digested by Fse I and Pml I (NEB, Ipswich, MA), yielding a 2.3kb fragment that included the intronic CArG element and surrounding loxP sites. The smMLCK^{-389~+8427} pWhere vector was also cut by Fse I and Pml I. The resulting 2kb fragment that included the intronic CArG element was replaced with the corresponding 2.3kb fragment isolated from the targeting vector to generate the I_{CArG}-smMLCK^{-389~+8427} pWhere plasmid. The integrity of the plasmid was confirmed by restriction enzyme digestion and DNA sequencing. In order to delete the CArG element from the I_{CArG}-smMLCK^{-389~+8427} pWhere plasmid, this plasmid was electroporated into SW106 bacteria (NCI-Frederick, MD) which has a Cre combinase (Cre) inducible gene. L-arabinose-induced Cre expression in SW106 cells at 32°C resulted in excision of the loxP flanked intronic CArG region to generate plasmid Δ I_{CArG}-smMLCK^{-389~+8427} pWhere. Correct excision of the CArG element was confirmed by DNA sequencing. I_{CArG}-smMLCK^{-389~+8427} pWhere and Δ I_{CArG}-smMLCK^{-389~+8427} pWhere plasmids were linearized and microinjected into pronuclei of fertilized oocytes by standard procedures by the Indiana University School of Medicine transgenic mouse facility. Neonatal founder mice were genotyped for the presence of the transgene and were analyzed at one month old by β -galactosidase staining as described previously [154]. All animal experiments were conducted under the approval of Indiana University School of Medicine Institutional Animal Care and Use Committee.

iii. Generation of knockout mice

The intronic CArG region floxed mouse was generated by inGenious Targeting Laboratory (Stony Brook, NY). The targeting construct was linearized using NotI (NEB, Ipswich, MA) prior to electroporation into C57BL/6N embryonic stem cells. Positively selected ES cells were screened by PCR and then expanded for southern blot confirmation of targeting. Correctly targeted ES cells were microinjected into BALB/c blastocysts. Resulting chimeras with a high percentage black coat color were mated to C57BL/6 Flp mice to remove the Neo cassette. The deletion of Neo cassette was screened and confirmed by PCR and DNA sequencing. The germline transmitted floxed mouse was shipped to Indiana University School of Medicine Laboratory Animal Resource Center (LARC) and quarantined for two months. Then the floxed mouse was mated to smooth muscle myosin heavy chain driven Cre mouse (smMHC-Cre mouse) that was kindly provided from Dr. Michael Kotlikoff at Cornell University, to delete the intronic CArG region specifically in smooth muscle tissues. Genotyping primers for the floxed mice are: flox F: GGC AAG CCA AAC CCT TAC ACA G, flox R: GAC TGG AGA TAA CCT CCT CTC ACT

iv. Contractility measurement of isolated colon rings

Colon was isolated and cleaned gently. The proximal part of the colon was cut into rings about 0.5 cm in length and mounted onto steel supports, submerged in a 5-ml organ bath with physiological saline solution (PSS) saturated with 95%

O₂-5% CO₂ at 37°C, as described previously [155]. PSS solution contains 119 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 2 mM CaCl₂, 1.17 mM KH₂PO₄, 1.17 mM MgSO₄, and 5.5 mM glucose, pH 7.4. A 1.5-g preload passive tension on these colon rings was empirically determined to result in the optimal contraction after 60 mM KCl (P9541, Sigma-Aldrich, St. Louis, MO) stimulation. The passive tension was increased gradually in half-gram increment until 1.5g, and colon rings were incubated in the bath system to equilibrate for about one hour until stabilized. Then they were challenged with 60 mM KCl or 1 µM carbachol (C4382, Sigma-Aldrich, St. Louis, MO), a muscarinic agonist, to induce colon smooth muscle contraction. To determine the effect of calcium channel blocker on the induced contraction, L-type calcium channel inhibitor diltiazem (1µM, A2521, Sigma-Aldrich, St. Louis, MO) was administered to the organ bath for 5 minutes before high KCl and carbachol stimulation.

v. Contractility measurement of isolated thoracic aorta

Thoracic aortas (about 0.5 cm) were dissected carefully and their branches were ligated with thread under microscope before being used. The aortas were mounted in the chamber filled with PSS aerated with mixed gas (22% O₂-5% CO₂-73% N₂). The aorta was bridged to a PSS-filled tube that was pressurized with a regulator to inflate the vessels to the desired pressure before chemical stimulation. A pressure transducer (SPR-524, Microtip catheter transducer, Millar, Houston, TX) was used to monitor the intraluminal pressure, and a volume

compensator used to compensate for water transport across the vessel wall. A CCD camera on the microscope was able to convert the real-time image of the vessels to the computer for better visualization. The vascular contraction during endothelin 1 (ET1) stimulation was recorded as changes in intra-luminal pressure and quantitated using dimensional analysis software (DIAMTRAK 3, Australia) [156].

vi. Quantitative RT-PCR

Total RNAs were extracted from colon smooth muscle, bladder and thoracic aorta of 6 weeks old control and smooth muscle-specific 130kDa MLCK knockout mice. The mRNA expression levels were quantitated by reverse transcription-qPCR as described previously [157]. Because the 130kDa smMLCK transcript has a unique 5' UTR region not present in the 220kDa MLCK transcript, we were able to take advantage of the unique region to design primers specifically detecting the 130kDa smMLCK mRNA. The primers used for the detection of 220kDa MLCK, 130kDa MLCK and telokin are: 220kDa MLCK F: GAA CCT CTG CAT CAA AGA AGG AG, 220 MLCK R: GAT GGC TTG CCC TTT TCT GTG CCA TG, 130kDa smMLCK F: CTC TTG CTA CTT TCT CTT TTT CCT TCA CTG, 130kDa smMLCK R: CTG GTC TCC ACC CGT CTC TTC AAC AG, Telokin F: GAC ACC GCC TGA GTC CAA CCT CCG, Telokin R: GGC TTT TCC TCA GCA ACA GCC TCC

vii. Western blot analysis

Colon and bladder smooth muscle from both control and knockout mice were homogenized in the ice-cold glass tissue grind tube using the paired pestle. Total protein lysates from colon smooth muscle and bladder were extracted using radioimmune precipitation assay (RIPA) lysis buffer and protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA), as described previously [157]. 15µg of proteins was fractionated on 7.5% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Antibodies used for detecting MLCK were a polyclonal antibody raised against the common carboxyl-terminus of MLCK and telokin (CT polyclonal, 1:5000) [158] and a polyclonal antibody raised against the full length bovine smMLCK (FL polyclonal, 1:1000) [159]. Vinculin was used as loading control (V4505, clone VIN-11–5, Sigma-Aldrich). Horseradish peroxide (HRP) conjugated goat anti-rabbit secondary antibody (1:10,000) was used to detect and amplify the first antibody. Signal was developed using homemade enhanced chemiluminescence solution (ECL) and quantitated on a G-box imaging machine (Syngene, Cambridge, UK).

viii. MLC phosphorylation

The proximal portion of the colon was cut into 0.5 cm-long circular rings, and the rings were hung in an organ bath, as described above for contractility

measurements of colon. Tissues were clamp flash frozen in liquid nitrogen at the basal non-contracted status or at the peak of contraction induced by 60 mM KCl and incubated in dry iced pre-cooled acetone denature buffer with dithiothreitol (DTT) and trichloroacetic acid (TCA) for one hour. Then the dried tissues were minced and proteins were extracted by vortexing at low speed in urea gel extraction buffer containing 8 M urea, 20 mM Tris base, 22 mM glycine, and 10 mM dithiothreitol. 15 μ l of the extracts were loaded onto 10% urea/glycerol gel to separate proteins for overnight. The bands for both unphosphorylated and phosphorylated myosin light chain (MLC) were visualized by Western blotting and the ratio of phosphorylated to total MLC was quantitated, as described previously [160].

ix. Cell proliferation

The intestines of littermate neonatal mice (day 9-10) were dissected and the lowest portions of the ileum were incubated in 20% sucrose in PBS solution overnight at 4°C. Tissue samples were frozen into Tissue-Tek O.C.T. compound (Cat#4583, Sakura, Netherlands) and 7 μ m sections were cut. Sections were fixed with 3.7% formaldehyde, permeabilized in 0.2% triton X-100 and blocked with 10% FCS in 50mM Tris pH7.6, 150mM NaCl, then incubated with antibodies against Ki67 (Cat#15580, Abcam, Cambridge, UK, 1:500) and smooth muscle α -actin (Cat#A2547, Sigma-Aldrich, St. Louis, MO, 1:500). Primary antibodies were visualized by incubation with rhodamine-conjugated Goat anti-Rabbit IgG

(Jackson ImmunoResearch, West Grove, PA, 1:50) and FITC-conjugated Donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, 1:50) secondary antibodies. The staining of the sections was observed under fluorescent microscope (Nikon, Tokyo).

x. Statistical analysis

Statistical comparison was performed by student's t-test (Prism, GraphPad Software, La Jolla, CA). A value of $p < 0.05$ was considered statistically significant.

D. Results

i. Deletion of an intronic CArG element in the *mylk1* gene abolished transgene expression driven by the 130kDa smMLCK promoter.

Previously we reported a highly conserved CArG element located in intron 15 of the *mylk1* gene (1st intron of the 130kDa smMLCK) [128]. To investigate the role of this intronic region in regulating 130kDa smMLCK gene expression, we generated transgenic mice in which a lacZ reporter gene was driven by the 130kDa smMLCK promoter, exon1, intron1 and a portion of exon 2 with (I_{CArG}-smMLCK^{-389~+8427} pWhere) or without (Δ I_{CArG}-smMLCK^{-389~+8427} pWhere) this intronic CArG region, as described in Methods. In 2 of the 3 independent founders harboring the wild type transgene, high levels of β -galactosidase staining were observed in visceral smooth muscle rich tissues, such as bladder, colon, small intestine and ureters (Figure 8). The third line had lower levels of expression but in a similar pattern (not shown). One of the 2 high expressing founders also exhibited staining in bronchi as well as in the lung and small vessels of skeletal muscle and liver (Figure 8).

In contrast to the wild type transgenes, no β -galactosidase expression could be detected in any of 7 founder mice harboring the CArG deleted transgene (Figure 8). Weak background staining seen in bladder, kidney and colon is similar to that seen in non-transgenic mice (Figure 8, lower right panel). These data

demonstrate that the intronic CArG region is critical for expression of a 130kDa smMLCK-driven transgene.

ii. Deletion of the intronic CArG region from the endogenous *mylk1* gene resulted in decreased expression of the 130kDa smMLCK.

To determine if deletion of the intronic CArG region would decrease expression of the endogenous 130kDa smMLCK, we crossed mice harboring the floxed CArG region with mice expressing cre recombinase under the control of the smooth muscle myosin heavy chain promoter (Figure 9) [161]. Both control (global heterozygous) and smooth muscle-specific CArG knockout mice reached adulthood without any obvious growth and behavioral abnormalities. However, knockout mice were born with a lower than expected frequency (19% versus 25%, 50 out of 262, Figure 9C). This suggests that deletion of the intronic CArG region from the native gene results in partial embryonic lethality or neonatal death.

Quantitative real-time RT-PCR showed that there was an approximately 40% decrease in 130kDa smMLCK mRNA levels in both colon and bladder of knockout mice, compared with control mice, while there was no significant alteration in 220kDa MLCK or telokin mRNA expression levels (Figure 10A). In addition, there was an approximately 30% reduction of 130kDa smMLCK mRNA levels in the aorta of knockout mice. Moreover, using an antibody to the common

carboxyl-terminus of MLCK and telokin, we found that deletion of the intronic CArG region reduced 130kDa smMLCK protein expression by approximately 30% and 40% in colon and bladder, respectively (Figure 10B,C). Similar findings were observed using an MLCK antibody raised against the full-length bovine smMLCK (Figure 10C). Additional experiments confirmed that the presence of the loxP sites in the control mice did not alter 130kDa smMLCK expression compared to wild type mice (data not shown).

iii. Deletion of the intronic region and subsequent decreases in 130kDa smMLCK expression attenuated smooth muscle contractility.

In order to investigate whether the decreased expression of the 130kDa smMLCK affects the contractility of both visceral and vascular smooth muscle, we analyzed the contractility of colon and aortic segments, *ex vivo*. Contraction elicited by high KCl-induced depolarization of colon from knockout mice was dramatically decreased compared with control mice (Figure 11A,C). Similarly carbachol induced contractions were also impaired in tissue from knockout mice (Figure 11B,C). L-type calcium channel inhibitor diltiazem markedly inhibited the contractile responses to both high KCl and carbachol (data not shown).

ET1-mediated contraction of aortic segments was also decreased in knockout mice compared with control (Figure 11D) after a high concentration of ET1 stimulation.

iv. Decreased contraction of smooth muscle tissues in knockout mice was associated with decreased myosin light chain phosphorylation.

The 130kDa smMLCK induces contraction of smooth muscle by phosphorylating the regulatory myosin light chain. Thus we sought to determine if the impaired contractile responses seen in the knockout mice were associated with altered myosin light chain phosphorylation. Under basal resting conditions, levels of MLC phosphorylation in both control and knockout mice were very low and showed no significant difference (Figure 12). However, at the peak of contraction induced by high KCl, the level of phosphorylation of the MLC in knockout mice was much less than that seen in control mice (18.3% as compared to 39.3%, Figure 12).

v. Decreased 130kDa smMLCK expression resulted in shortened small intestine.

Further analysis of both control and knockout mice showed that the length of small intestine was shorter by about 24% in the intronic CArG knockout mice, while the length of colon was not significantly different (Figure 13A, B). Consistent with this observation, immunofluorescence staining of cross-sections of ileum from neonatal mice for the cell proliferation marker Ki67 revealed that there were fewer positively stained smooth muscle cells in both circular and longitudinal smooth muscle layers of knockout mice (Figure 13C,D). Interestingly,

preliminary data showed there was more positive smooth muscle Ki67 staining in the ileum than that in colon or upper part of small intestine, suggesting it is likely after birth the ileum plays a major role in mediating the elongation of intestine.

E. Discussion

Results of this study clearly show that a CArG box-containing region in intron 15 of the *mylk1* gene is required for expression of the 130kDa smMLCK. Deletion of this intronic CArG box attenuates expression of the 130kDa smMLCK without affecting expression of either the 220kDa MLCK or telokin. Moreover, decreased expression of only the 130kDa smMLCK in smooth muscle tissues attenuates smooth muscle contractility associated with decreased myosin light chain phosphorylation and impairs small intestine smooth muscle proliferation. This demonstrates that the 130kDa smMLCK isoform is specifically required to regulate not only smooth muscle contraction, but also intestinal smooth muscle cell proliferation.

Deletion of the intronic CArG region completely abrogated expression of an smMLCK-lacZ reporter transgene in the visceral smooth muscle tissues, whereas deletion of this element from the endogenous gene only decreased endogenous 130kDa smMLCK expression by about 40%. This would suggest that the reporter transgene is perhaps missing additional positive cis-acting regulatory elements that play a role in regulating expression of the endogenous 130kDa smMLCK. In support of this possibility a notch responsive element has been identified at -3687bp upstream of transcription start site and plays an important role in activating 130kDa smMLCK expression in vascular smooth muscle cells [162]. This element is not present in the reporter genes described in our study, which

extend from -389 to +8427. This may also explain why the reporter genes exhibited very low levels, or undetectable, LacZ expression in vascular smooth muscle tissues (Figure 9). Although the endogenous 130kDa smMLCK is expressed at lower levels in many nonmuscle tissues, we did not observe significant levels of lacZ transgene expression in many of these tissues. This may simply reflect the sensitivity of lacZ detection, although this may also reflect the nonnative chromatin environment of reporter transgenes. Although the transgenic reporter mice were generated using a pWhere lacZ expressing vector that is CpG-free and has H19 insulator elements flanking the transgene, we have previously shown that the telokin promoter also does not drive high levels of expression in many founder mice generated using this transgene vector [154]. As the promoter and regulatory elements analyzed are embedded within introns of the larger *mylk1* gene it is possible that transcription from the promoters that drive expression of the 220kDa MLCK may modulate the chromatin structure of the gene to facilitate the activity of these internal elements. Although analysis of reporter genes suggest that the promoter and first intron of the 130kDa smMLCK are not sufficient to fully recapitulate expression of the endogenous 130kDa MLCK, deletion of the intronic CArG region from the endogenous gene decreases 130kDa smMLCK expression by 40%, demonstrating that this element is required for full activation of the gene. The region deleted following Cre recombinase mediated recombination of the lacZ reporter or the endogenous *mylk1* gene includes a conserved CArG box together with almost 300bp of flanking sequence, We have previously shown that the CArG element is located

within a region of 63bp that is highly conserved between species [128]. This raises the possibility that the decreased 130kDa smMLCK seen following deletion of this region may be due to loss of not only SRF binding but also other as yet unidentified transcription factors.

Through analysis of transgenic reporter mice and targeting the endogenous telokin promoter, we have previously shown that a CArG element within the telokin promoter is also critical for expression of telokin transcripts [163,164]. Together with the current findings these data show that SRF plays a key role in regulating expression of multiple transcripts from the *mylk1* gene. Although SRF is important for regulating expression of both 130kDa smMLCK and telokin transcripts, it does so by binding to distinct CArG elements. These elements appear to be functionally separated from each other as deletion of a single CArG element affects expression of one transcript but not the other. Deletion of the CArG element in the telokin promoter (in *mylk* intron 28) abolished telokin expression without affecting expression of transcripts encoding the 200kDa or 130kDa MLCKs [163]. Similarly, deletion of the CArG element from intron 1 of the 130kDa smMLCK gene (in *mylk1* intron 15) decreased expression of the 130kDa smMLCK without affecting expression of the 220kDa MLCK or telokin (Figure 10). These data would suggest that either the CArG elements are simply too far from the other promoters to affect their activity (e.g. the telokin CArG element is about 73kb from the 130kDa smMLCK promoter) or that there are perhaps insulator elements within the *mylk1* gene that restrict the activity of the elements to

specific promoters. Additional studies will be required to resolve these possibilities.

Previous studies have shown that deletion of both the 220kDa and 130kDa MLCK from smooth muscle tissues impairs contractility, myosin light chain phosphorylation and impairs gastrointestinal motility in mice [150]. The current studies suggest that it is primarily the 130kDa smMLCK rather than the 220kDa MLCK that is responsible for regulating contraction in gastrointestinal smooth muscle. Results also suggest that the 130kDa smMLCK has a specific role in regulating the proliferation of small intestinal smooth muscle cells during early neonatal growth (Figure 13). Although MLCK and myosin light chain phosphorylation are known to be important in cell division this is the first data that demonstrate a specific role for the 130kDa smMLCK isoform in this process.

In summary, data from both transgenic reporter mice and knockout mice models, demonstrate that a CArG region within intron 15 of the *mylk1* gene plays an important role in specifically regulating expression of 130kDa smMLCK. The I_{CArG} flox mice provide a novel model system for further interrogating the specific functions of the 130kDa smMLCK isoform in different cell types *in vivo*.

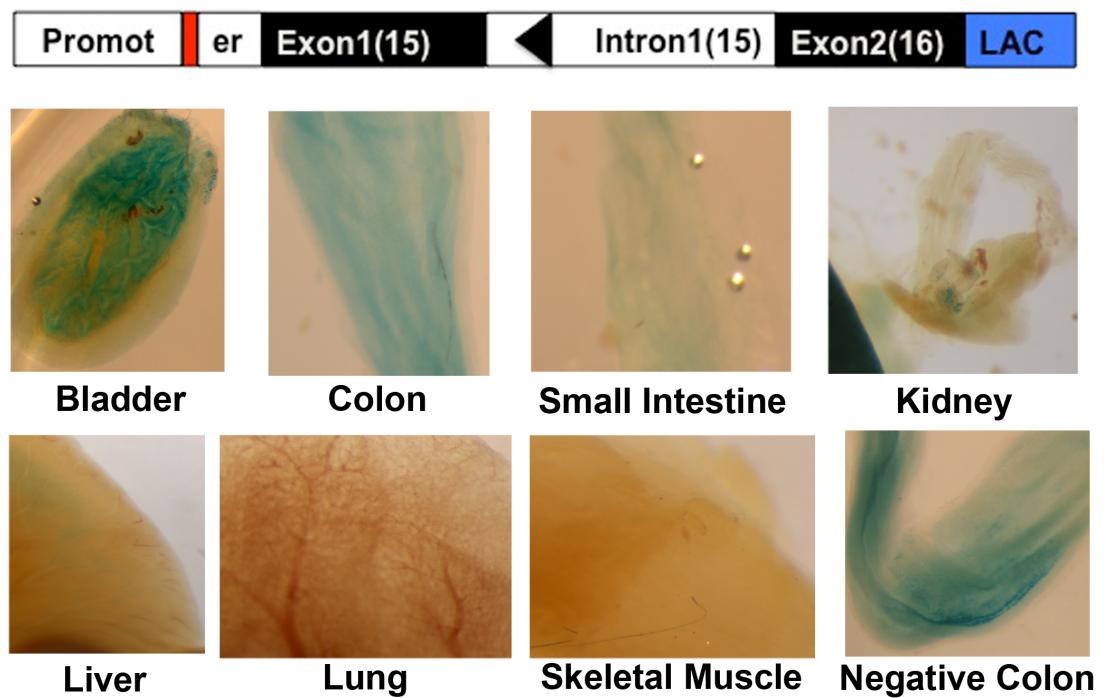
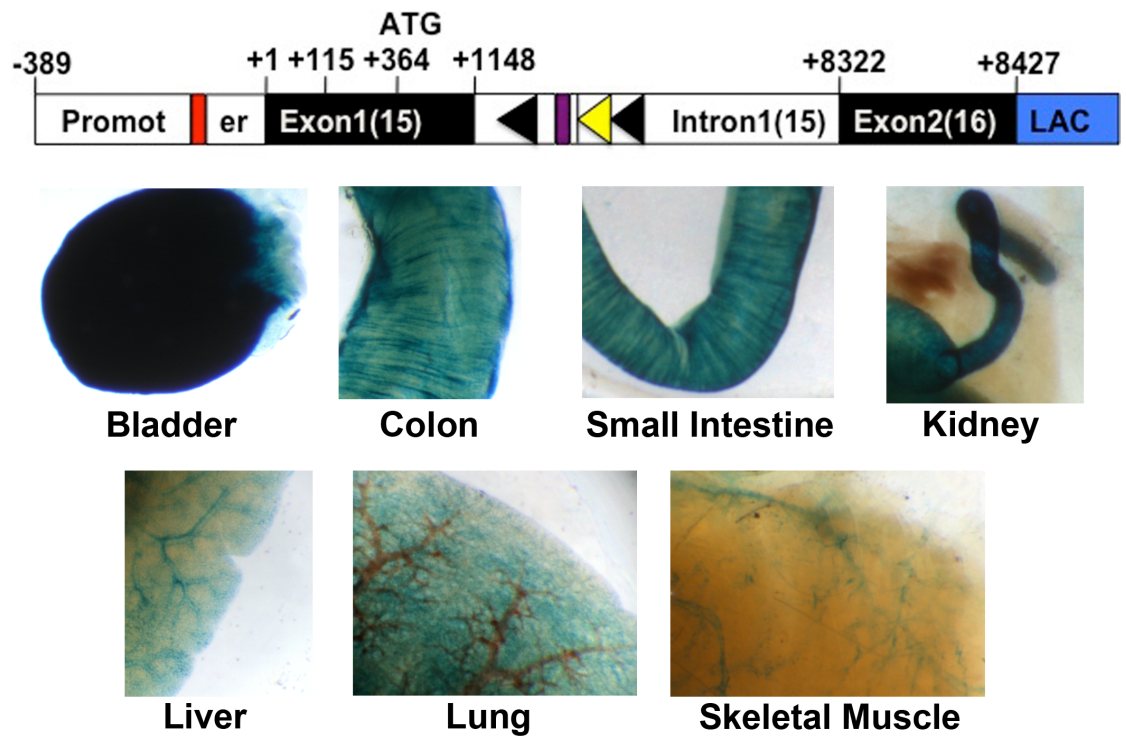


Figure 8. Comparison of transgene expression in different reporter mice

A. Organs were harvested from 1-month old reporter mice harboring the $I_{\text{CArG}}\text{-smMLCK}^{-389\sim+8427}$ pWhere transgene (shown schematically at the top) and lacZ expression was examined by X-Gal staining (blue/green color, n=3). Yellow triangle, loxP site; black triangle, FRT site; blue box, intronic CArG element; red box, promoter CArG element.

B. β -galactosidase expression in organs harvested from 1 month old mice harboring the $\Delta I_{\text{CArG}}\text{-smMLCK}^{-389\sim+8427}$ pWhere transgene (schematic, upper panel) in which the intronic CArG element is deleted (images are representative of 7 founder mice). Lower right panel shows β -galactosidase staining of colon from a nontransgenic mouse as a negative control (Negative colon).

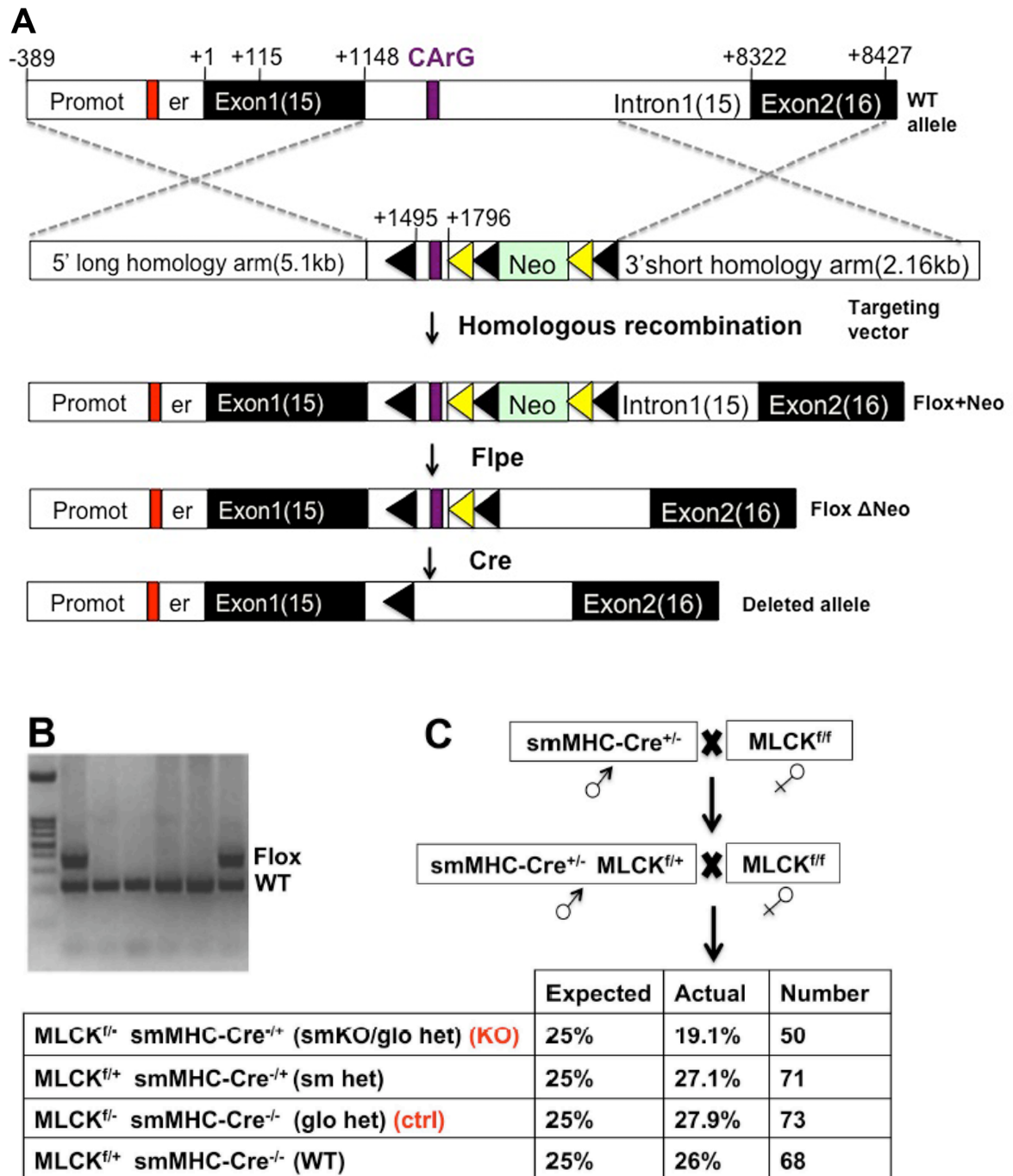


Figure 9. Generation and genotyping of the intronic CArG knockout mice

A. Schematic representation of approach used to delete the intronic CArG region from the endogenous *mylk1* gene. The native *mylk1* gene is shown at the top

with the targeting vector below it. Numbers above the native gene refer to nucleotides positions relative to the transcription start site of the 130kDa smMLCK. The promoter and intronic CArG boxes are indicated (red and blue boxes, respectively). Yellow triangles, loxP sites; black triangles Frt sites. Below the targeting vector are schematic representations of the correctly targeted allele (Flox+Neo), the targeted allele following Flpe-mediated removal of the neomycin cassette (Flox) and the deleted allele generated following cre-mediated recombination (Deleted allele).

B. Ethidium bromide stained agarose gel showing an example of the genotyping of the Flox mice using primers indicated by the arrows in panel A.

C. Breeding scheme used to generate knockout mice together with the genotypes of the progeny, their expected frequency, observed frequency and total numbers of pups analyzed.

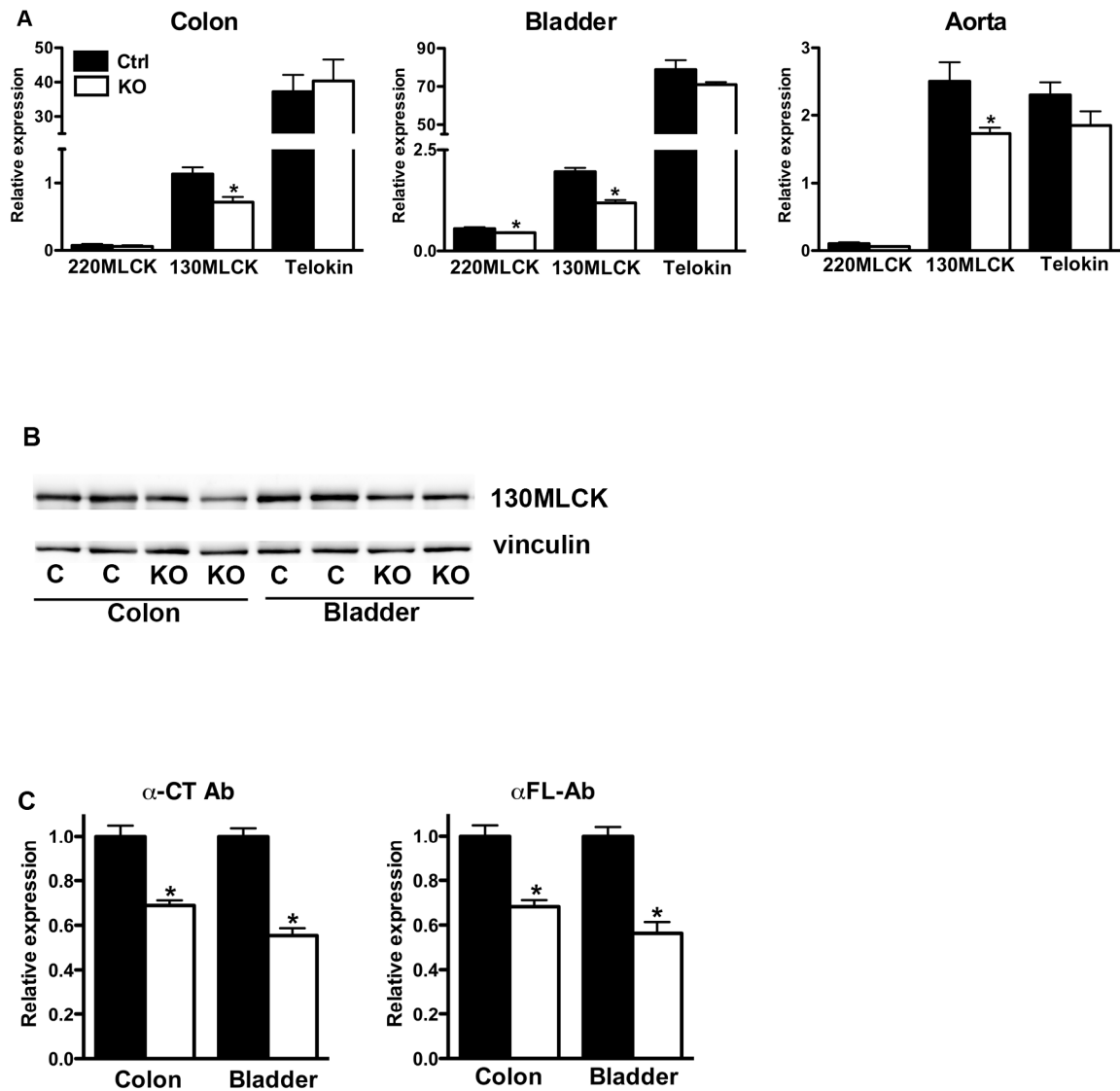


Figure 10. The expression of the 130kDa smMLCK is decreased in CArG knockout mice

Smooth muscle layers were isolated from the indicated tissues of control (Ctrl) and knockout (KO) mice and total RNA or protein were harvested.

A. The mRNA levels in colon, bladder and thoracic aorta from control and knockout mice were measured by qRT-PCR. Transcript levels were normalized to hprt internal loading control and relative expression levels (RQ) are shown.

Relative expression = $2^{-\Delta\text{DCt}}$, where $\Delta\text{DCt} = (\text{Ct}_{\text{KO expt}} - \text{Ct}_{\text{KO hpert}}) - (\text{Ct}_{\text{Ctrl expt}} - \text{Ct}_{\text{Ctrl hpert}})$. Each column represents the mean \pm SEM of samples obtained from 11-13 mice. *, $p < 0.05$.

B. Representative western blot result of 130kDa smMLCK and vinculin in colon and bladder from two control (C) and two knockout (KO) mice.

C. Quantitation of western blots using different antibodies for smMLCK. Data were normalized to Vinculin levels and are expressed relative to expression levels in control mice. CT Ab: a polyclonal antibody raised against the common carboxyl-terminus of MLCK and telokin, FL Ab: a polyclonal antibody raised against the full-length bovine smMLCK. $n=11-13$. *, $p < 0.05$.

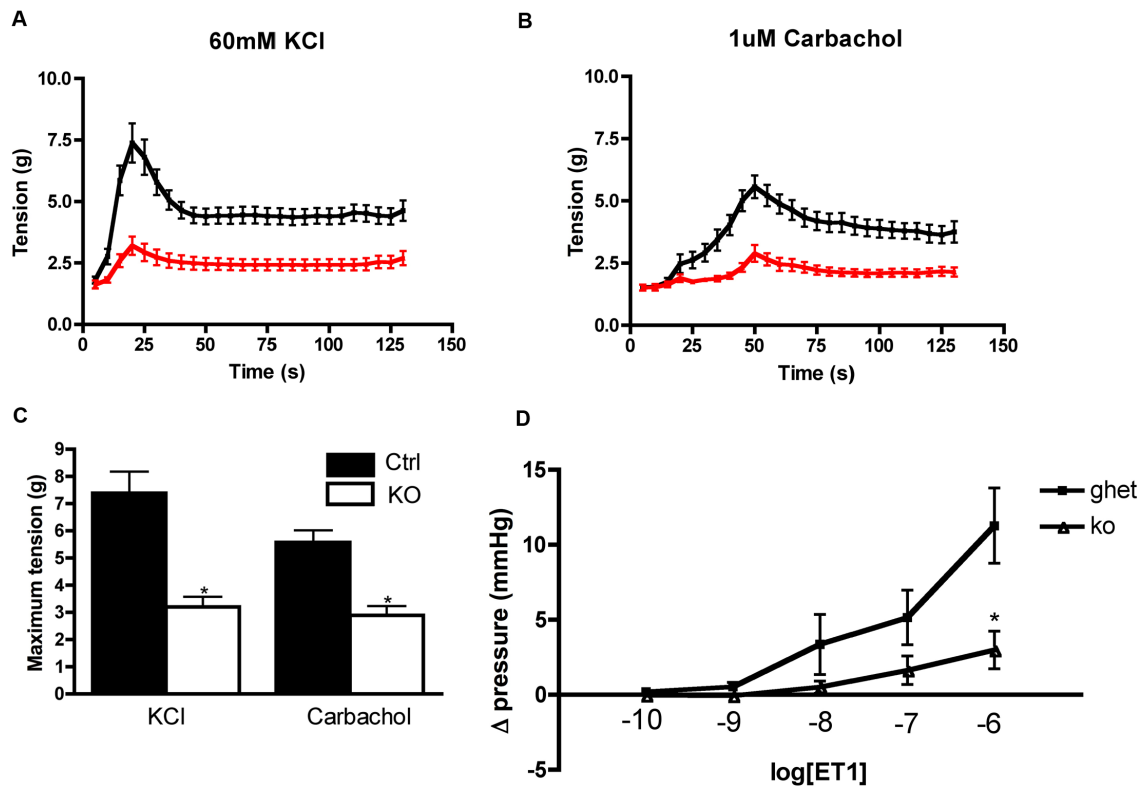


Figure 11. The contractility of smooth muscle tissues from knockout mice is decreased.

A-B. Colon rings were hung in an organ bath and stimulated to elicit contraction with 60mM KCl or 1μM Carbachol as described in methods. Data shown are the means \pm standard deviations in tension over time of 20 rings from control mice and 12 from knockout mice. Red color denotes knockout mice and black color denotes control mice.

C. The average changes in peak contractile responses of colonic rings from control (Ctrl) and knockout (KO) mice. n=20 for control, n=12 for knockout. *, p<0.05.

D. Mean maximal contractile responses of thoracic aortic to increasing doses (M) of Endothelin 1 (ET1). n=6 for control, n=5 for knockout. *, $p < 0.05$.

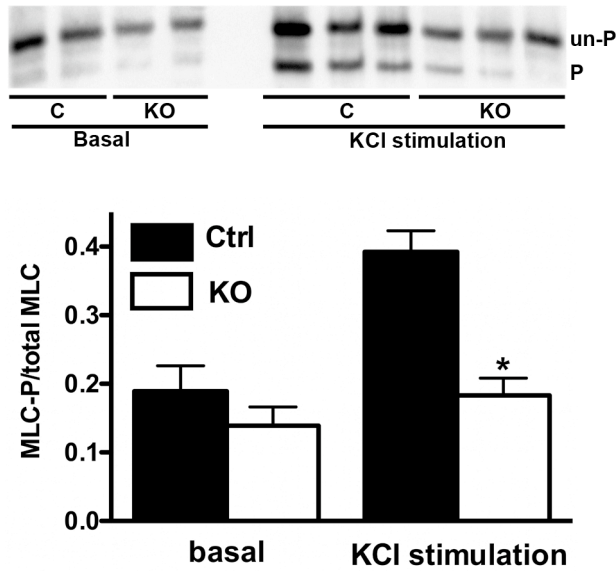


Figure 12. The myosin light chain phosphorylation is decreased in knockout mice.

Colon rings were either flash frozen under resting conditions or challenged by 60mM KCl and flash frozen at the peak of contraction. Unphosphorylated and phosphorylated myosin light chains were separated by urea/glycerol gel electrophoresis and visualized by western blotting.

A representative western blot is shown in the upper panel. (un-P, unphosphorylated MLC; P, phosphorylated MLC; C, control; KO, knockout).

The ratios of MLC-P to total MLC under basal conditions (n=6) and after KCl stimulation (n=10) were calculated and the mean values \pm SEM are plotted. *, p<0.05.

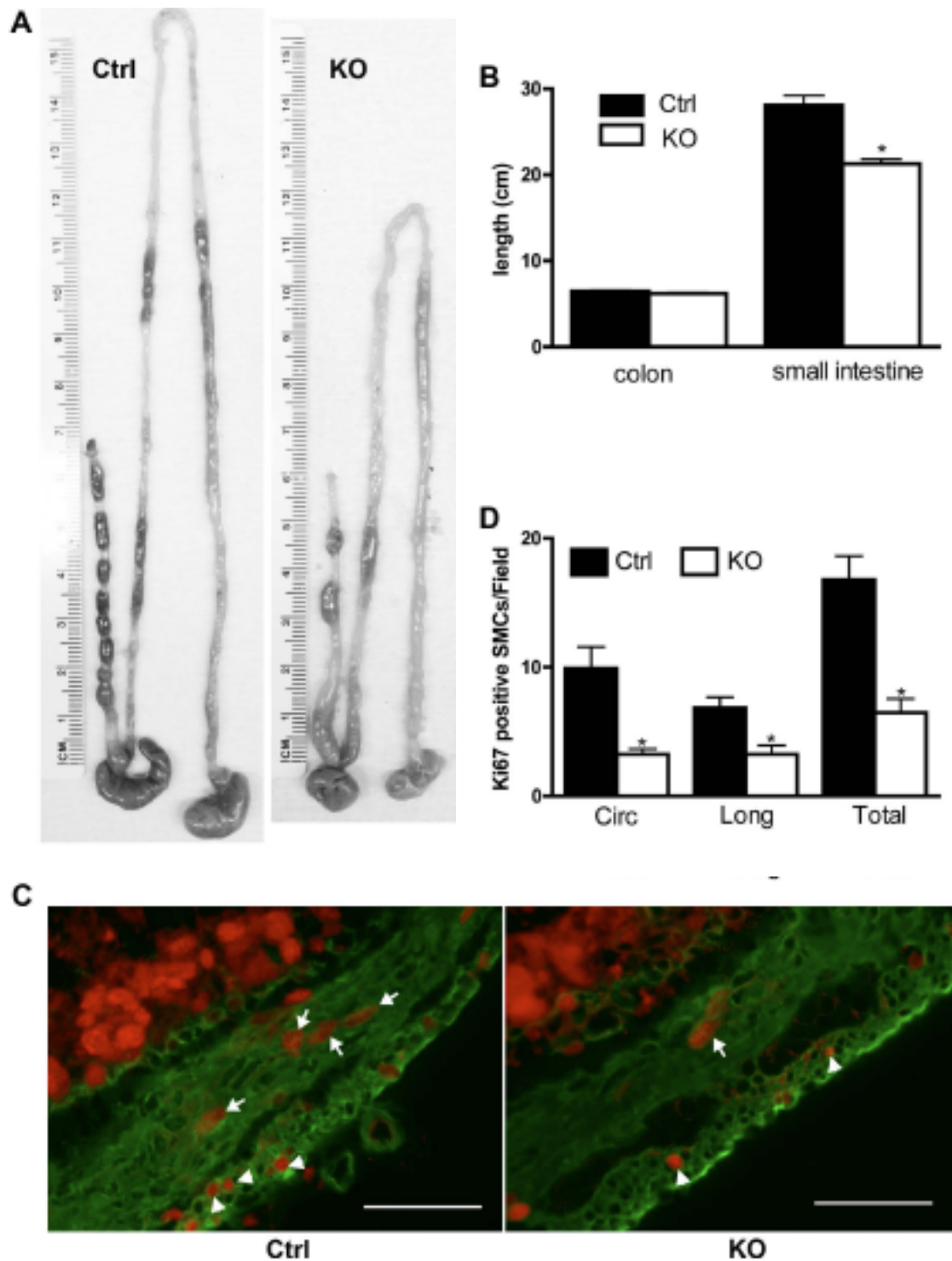


Figure 13. Knockout mice have decreased smooth muscle cell proliferation and shorter small intestines.

A. Representative pictures and B, quantitative data showing the lengths of colon and small intestine of adult control and knockout mice. n=5. *, p<0.05.

C. Ki67 (red) and smooth muscle α -actin (green) staining of cross sections of the lower portion of small intestines from neonatal control and knockout mice. White arrows and white arrow heads point to examples of Ki67 positive smooth muscle cells in the circular and longitudinal smooth muscle layers, respectively.

D. Quantitation of the number of positive Ki67 smooth muscle cells per field at 40x magnification in the circular layer, longitudinal layer and both smooth muscle layers of the small intestine. n=6-7 mice. *, p<0.05.

CHAPTER III

REGULATION OF MICRORNAS BY BRAHMA-RELATED GENE 1 IN SMOOTH MUSCLE CELLS

A. Summary

MicroRNAs are involved in the phenotypic switch between differentiation and proliferation of smooth muscle cells. Brg1-containing SWI/SNF chromatin remodeling complexes also play an important role in controlling the phenotype of smooth muscle cells. We thus determined if Brg1 influences the transcription of microRNAs in smooth muscle cells. Microarray and qRT-PCR analysis of smooth muscle from mice harboring smooth muscle-specific deletion of Brg1 revealed altered expression of several microRNAs, including microRNAs-143/145 (miRs-143/145) and miR-133. Dominant negative Brg1 attenuated miRs-143/145 expression in wild type smooth muscle cells *in vitro* and myocardin-induced miRs-143/145 expression in 10T1/2 cells. In Brg1 null SW13 cells, miRs-143/145 were dramatically induced by myocardin only in the presence of Brg1, while miRs-133 was not induced by myocardin in a Brg1-dependent manner. Knockdown of myocardin or serum response factor (SRF) in smooth muscle cells significantly reduced the expression levels of miRs-143/145 although miR-133 expression was only attenuated by SRF knockdown. Chromatin immunoprecipitation assays demonstrated that myocardin in the presence of Brg1, increased the binding of SRF to the miRs-143/145 promoter as well as the

regulatory regions of miR-133. Together these data suggest a mechanism in which Brg1-containing SWI/SNF complexes are required for myocardin to induce expression of miRs-143/145 in smooth muscle cells. In contrast, miR-133 expression appears to be regulated by Brg1-containing chromatin remodeling complexes in a partially SRF-dependent, although largely myocardin-independent fashion. These observations together with what we reported previously imply that SWI/SNF-mediated chromatin remodeling regulates the phenotype of smooth muscle not only by directly affecting expression of smooth muscle-specific protein coding genes, but also by regulating expression of microRNAs that further modulate expression of these genes.

B. Introduction

MicroRNAs are endogenous small non-coding RNAs (~22nt) which have emerged as key regulators of gene expression through inhibiting translation and/or promoting degradation of their mRNA targets. During microRNA biogenesis, primary microRNAs are first transcribed by RNA polymerase II from genes that lie either between or within protein coding genes. Primary microRNA transcripts are then sequentially processed by endonucleases Drosha and Dicer into mature microRNAs. MicroRNAs are widely expressed and exhibit tissue specific and dynamic expression patterns during development and pathophysiological processes.

The importance of microRNAs in smooth muscle cell (SMC) differentiation and function has been demonstrated through analysis of mice lacking Dicer in these cells [165] [166] [114]. Deletion of Dicer in mouse smooth muscle cells by an SM22 α promoter driven Cre transgene resulted in embryonic lethality at embryonic day 16 to 17 with underdeveloped vessels and extensive hemorrhage [165]. Deletion of Dicer in more mature smooth muscle cells mediated by smooth muscle myosin heavy chain (smMHC) driven Cre transgene did not result in embryonic lethality although the mice developed severe intestinal dysmotility with loss of the external smooth muscle layers [166]. Postnatal deletion of Dicer in SMCs using a tamoxifen regulated smMHC-Cre transgene resulted in a dramatic reduction of blood pressure and loss of contractile proteins within the vasculature

[114]. These studies demonstrate that microRNAs play a key role in the development and maintenance of SMCs in both vascular and gastrointestinal tissues.

Among Dicer-dependent microRNAs, the most abundant in smooth muscle cells are miRs-143/145. MiR-143 and miR-145 are two mature microRNAs that are encoded by the same bicistronic primary microRNA transcript [167]. Although miR-143 and miR-145 knockout mice are viable, they have significant reduction in blood pressure, thinner smooth muscle layers, incomplete differentiation of SMC and altered neointima formation in response to vascular injury [167,168,169]. MiRs-143 and 145 have been shown to cooperatively modulate a network of targets via feed-back, feed-forward, or double-negative feedback mechanisms [170]. For example, miRs-143/145 can target Kruppel-like factor 4 (Klf4), myocardin and member of ETS oncogene family (Elk1) [171], as well as versican [172] to promote differentiation and repress proliferation of smooth muscle cells. Moreover, several targets have been identified that regulate actin dynamics and cytoskeletal organization, such as myocardin-related transcription factor-B (MRTF-B), Adducin-3 (ADD3), Slingshot 2 phosphatase (Ssh2), Slit-Robo GTPase-activating protein 1 (Srgap1) and Srgap2 [167]. Using LacZ reporter mice, a 0.9-kb promoter region was shown to be sufficient to direct miRs-143/145 expression to cardiac and smooth muscle cells. Within this region, a highly conserved CArG element (SRF binding site) and Nkx2-5 binding region have been identified. Moreover, the miRs-143/145 gene is a direct transcriptional

target of SRF, myocardin and Nkx2-5, and it is up-regulated in more differentiated smooth muscle cells [171].

Recently, miR-133, which is conventionally considered as a cardiac or skeletal muscle specific microRNA, has also been shown to inhibit VSMC proliferation *in vitro* and after balloon injury *in vivo*, at least partially through regulating the expression of Sp1 and moesin [123]. In cardiomyocytes, deletion of miR-133 caused aberrant cardiomyocyte proliferation and ectopic expression of smooth muscle genes in the heart, partially through the up-regulation of miR-133 target genes SRF and cyclin D2 [173,174]. Moreover, Over-expression of miR-133 reduced cardiac hypertrophy while inhibiting miR-133 resulted in hypertrophy [175]. In skeletal muscle, miR-133 has been shown to repress myogenesis but promote proliferation, partially through targeting SRF [174]. As SRF is a key regulator for skeletal, cardiac and smooth muscle development, it is likely that miR-133 might be an important regulator of all three muscle lineages. The miR-133 isoforms miR-133a and miR-133b, are encoded by three genomic loci: miR-133a-1, miR-133a-2 and miR-133b. Each of these loci is transcribed as a bicistronic primary transcript containing one miR-133 isoform with another microRNA [176]. In skeletal muscle cells, expression of these microRNAs is regulated by the myogenic factors MyoD and myogenin, and in cardiac muscle miR-133a expression is regulated by MEF2 [176,177]. Although the miR-133 family members are now recognized as intriguing regulators of VSMC phenotypic modulation, little is known regarding the transcriptional regulation of these

microRNAs in smooth muscle cells. There is also little information on the epigenetic regulation of microRNAs in smooth muscle cells. In skeletal muscle cells, the Brg1 ATPase subunit of the SWI/SNF chromatin remodeling complex has been shown to be required for MyoD to activate transcription of both skeletal muscle contractile protein genes and miR-133a [127].

Previously, it has also been shown that Brg1 is required for myocardin or myocardin related transcription factors (MRTFs) such as MRTFA to induce expression of smooth muscle-specific contractile proteins [109,178]. In current study we investigated the role of Brg1 in regulating microRNA expression in smooth muscle cells. Our results demonstrate that several microRNAs, including miRs-143/145 and miR-133, are regulated by Brg1 in smooth muscle. Brg1 is required for myocardin to induce binding of SRF to the regulatory region of miRs-143/145, which is sufficient to activate its transcription. In contrast, the regulation of miR-133 expression by Brg1 requires more transcription factors than SRF itself.

C. Methods

i. Animal use

All the protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. To generate smooth muscle-specific Brg1 knockout mice, female Brg1^{flox/flox} mice (obtained from C.-P. Chang at Stanford University) [179] were bred with male smMHC-Cre/eGFP mice (from Michael Kotlikoff at Cornell University) [180]. Because of transient expression of Cre in the sperm of the male mice [181], the floxed allele transmitted from these male mice is recombined, thus there are four possible genotypes of the offsprings: Brg1f/- smMHC-Cre-/+ (SM-specific Brg1 knockout with global heterozygous Brg1 background; “smBrg1 knockout”), Brg1f/- smMHC-Cre-/- (global Brg1 heterozygous, which we use as a control for the knockout mice), Brg1f/+ smMHC-Cre-/+ (SM-specific Brg1 heterozygous), and Brg1f/+ smMHC-Cre-/- (wild type). Genotyping was performed as previously described using primer TG57 and primer TH185 for Brg1 floxed and wild-type alleles, giving rise to the PCR products of 387bp and 241bp, respectively. Primer TG57: GCC TTG TCT CAA ACT GAT AAG, primer TH185: GTC ATA CTT ATG TCA TAG CC. Primers for Cre are: Cre F: CAT TTG GGC CAG CTA AAC AT, Cre R: CCC GGC AAA ACA GGT GTT A [182]. The global heterozygous Brg1 mice (Brg1^{flox/-}) were used as control mice, while smooth muscle-specific Brg1-

null mice (smBrg1^{-/-}) with global heterozygous Brg1 background were the experimental smBrg1 knockout mice.

ii. Tissue harvest and RNA extraction

Two months old control and knockout mice were sacrificed. Colons and bladders were dissected quickly and were put in cold PBS buffer on ice. The colon was cleaned, cut open longitudinally and then the epithelial layer removed by scraping with a scalpel. Bladder was washed in ice cold PBS. All the tissues were then frozen in liquid nitrogen immediately. To extract RNA from tissues, samples were first pulverized in liquid nitrogen and then homogenized in guanidinium isothiocyanate using a Polytron (Kinematica, Switzerland). Total RNA was extracted following standard protocols [183].

iii. MicroRNA microarray

Total RNA samples from colon smooth muscle tissues were checked for RNA integrity number (RIN) on Agilent Bioanalyzer (Agilent Biotechnology). Four samples from each group with RIN > 8 were then used for microarray analysis. Total RNA samples were labeled using the Genisphere FlashTag HSR kit (Genisphere, Hatfield, PA). The labeled samples were hybridized to Affymetrix GeneChip® miRNA arrays (Affymetrix, Santa Clara, CA). They were stained and washed using the standard microRNA protocol. Affymetrix GeneChip Command

Console Software (AGCC) was used to scan the arrays and generate CEL files. CEL files were imported into Affymetrix microRNA QC Tool to generate expression levels and detection calls. Probe sets that were absent in more than two samples for both controls and knockouts were removed prior to importing the expression levels into Partek Genomics Suite (Partek, St. Louis, MO) for analysis [184]. A t-test was performed using the log base 2 transformation of the expression levels. Fold changes were calculated using the raw expression levels.

iv. Primary smooth muscle cell culture

Primary colon smooth muscle cells were isolated from 1-month-old wild type C57/B6 mice. After colons were dissected, cleaned, and cut open, epithelial layers were removed by scraping. The smooth muscle layers were then minced in Hanks buffered saline solution (HBSS) on ice and digested with 0.6 U/ml Liberase TM (Roche, Indianapolis, IN) and 0.25 mg/ml DNase I (NEB, Ipswich, MA) in HBSS at 37°C for 45 minutes with shaking. The digested cells were filtered through a 100µm filter, washed in SMC growth medium (containing Dulbecco's modified Eagle's medium DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin). Washed cells were plated in 6-well plates (1 well per colon) or SMC maintenance medium (containing Dulbecco's modified Eagle's medium DMEM supplemented with 1% FBS, 2% chick extract, 1% N2, 2% B27, 20ng/ml mouse bFGF, 100nM retinoic acid, 50nM β-mercaptoethanol, 50 units/ml penicillin, and 50 µg/ml streptomycin

and the medium was changed once per day until cells reached 100% confluence (usually about a week). Confluent primary cells were collected by trypsin digestion and plated in 6 or 12-well plates for adenoviral transduction.

v. Expression plasmids and adenoviral transduction

Human Brg1, Brm and dominant negative-Brg1 (DN-Brg1) plasmids were purchased from AddGene. MRTFA cDNA was purchased from Invitrogen. Mouse myocardin pcDNA3.1-myc/His vector was kindly provided by Dr. Eric N. Olson (UT Southwestern Medical Center, Dallas, TX). These plasmids were used to generate adenoviral expression vectors and adenoviral transductions were performed as described previously [109,178]. Adenovirus encoding nuclear localized yellow fluorescent protein (YFP) was used as negative control.

vi. Quantitative RT-PCR

Total RNA was extracted using Trizol reagent. MicroRNAs were quantitated using the small RNA quantitation system (SBI System Biosciences, Mountain View, CA) with some modifications. PolyA tails were added to the RNA by incubation with polyA polymerase at 37°C for 30 minutes. An oligo dT adaptor conjugated with a sequence complimentary to a universal reverse primer (CGA ATT CTA GAG CTC GAG GCA GG) was annealed to polyA-tailed RNAs at 65°C for 10 minutes followed by chilling on ice for 10 minutes. Then, cDNA was

synthesized and diluted as template for qPCR using microRNA specific forward primers (miR-143 forward primer: TGA GAT GAA GCA CTG TAG CTC, miR-145 forward primer: GTC CAG TTT TCC CAG GAA TCC CT, miR-133a forward primer: TTT GGT CCC CTT CAA CCA GCT G, miR-133b forward primer: TTT GGT CCC CTT CAA CCA GCT A) and universal reverse primer. U6 snRNA was used as internal control. (Human U6 snRNA control forward primer sequence: CGC AAG GAT GAC ACG CAA ATT C, mouse U6 snRNA control forward primer sequence: TGG CCC CTG CGC AAG GAT G). The mRNA expression levels were quantitated through reverse transcription-qPCR as described previously.

vii. Western blotting analysis

Proteins were extracted with RIPA lysis buffer on ice. Protein concentrations were determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA). 20µg of proteins were fractionated on 7.5 or 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were then probed with a series of primary antibodies. Antibodies used for Western blotting were: β -actin antibody (Sigma, St. Louis, MO, 1:10,000), Flag tag antibody (Sigma, St. Louis, MO, 1:5,000) for exogenous Brg1 and Brm, HA tag antibody (M2, Covance, Princeton, NJ, 1:3,000) for exogenous MRTFA, Omni antibody (M-21, sc-499, Santa Cruz, Dallas, TX, 1:3,000) for myocardin. Primary antibodies were then detected using horseradish peroxidase-conjugated secondary antibodies and visualized using chemiluminescence.

Chemiluminescent signals were collected and analyzed on a G-Box imager (Syngene, Cambridge, UK).

viii. Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays using cells were performed as described [178]. Briefly, cells were fixed in 1% formaldehyde for 10 min at room temperature and harvested using cold PBS with protease inhibitors. After collecting cells by centrifugation, cell pellets were lysed using 1% SDS lysis buffer with protease inhibitors (200 μ l lysis buffer/ 10^6 cells). For each group, 1 ml of lysate was sonicated at setting High with 30s burst and 30s silence for 9 cycles in a bioruptor (Diagenode, Belgium) at 4°C. The protocol for smooth muscle tissue chromatin immunoprecipitation assays was modified based on the one for cells. Specifically, tissues were finely minced before fixation. And the sonication condition for tissues was 45 cycles with 30s burst and 30s silence for each cycle. 200 μ l aliquots of chromatin were immunoprecipitated using 6 μ g of anti-SRF antibody (Santa Cruz, Dallas, TX), or rabbit IgG as negative control. The precipitated genomic DNA was purified and the presence of specific promoters was detected by real-time quantitative PCR, using gene promoter-specific primers. Primers for the telokin promoter were used as positive control. Primers used were shown in Table 1.

D. Results

i. Altered microRNA expression in smooth muscle tissues from smBrg1 knockout mice.

To determine which microRNAs are regulated by Brg1, we analyzed microRNA expression in smooth muscle tissues of control and smBrg1 knockout mice using Affymetrix GeneChip microRNA arrays. This analysis identified several microRNAs with decreased expression levels in knockout tissues, including miR-133a, 133b and miR-206. Several microRNAs including miR-423-5p, 423-3p, 34a, 28, 212, 674-5p exhibited increased expression in Brg1 knockout smooth muscle (>2-fold at a false discovery rate of <0.2). We did not observe a large change in expression of miR-143/145 in the array analysis, although miR-143 was decreased about 1.4-fold.

Quantitative RT-PCR analysis of mature microRNA expression levels was used to confirm the microarray results. This approach revealed that in colonic smooth muscle tissues of smBrg1 knockout mice there was a significantly lower level of expression of miR-143, 145, 133a, 133b, 30a-3p as compared to tissues from control mice, while miR-34a, 28, 674-5p were significantly up-regulated (Figure 14A).

Similar decreases in miR-143, 145, 133a, 133b were seen in bladder smooth muscle while only miR-28 was increased in bladders of smBrg1 knockout mice (Figure 14A). As miR-143, 145, 133a and 133b were decreased in both colon and bladder and these were the most abundant microRNAs analyzed in these tissues (Figure 14B), we focused on determining how Brg1 regulates the expression of these four microRNAs.

ii. Brg1 regulates the expression of miRs-143/145 in smooth muscle cells.

Altered microRNA expression observed in the tissues of smBrg1 KO mice could be a consequence of the pathological changes that occur in these mice as oppose to direct regulation of microRNA expression by Brg1. To distinguish between these possibilities, we introduced a dominant negative form of Brg1 (K798R) (DN-Brg1) directly into cultured primary smooth muscle cells by adenoviral transduction. Consistent with a previous report [178], the DN-Brg1 attenuated expression of smooth muscle markers such as smMHC, telokin, calponin (Figure 15A). The DN-Brg1 also attenuated expression of miRs-143/145, suggesting miRs-143/145 may be directly regulated by Brg1 (Figure 15B). Surprisingly, expression of miR-133a and 133b was not significantly affected by DN-Brg1 (Figure 15A).

To further confirm the results, we cultured the primary colon SMCs from Brg1^{flox/flox} mice and infected them with Cre-expressing adenovirus to delete Brg1

in vitro. After Brg1 was successfully deleted in primary SMCs, miRs-143/145 were significantly decreased while not for miRs-133a/b (Figure 15B). These findings suggest that the attenuated expression of miR-133 observed *in vivo*, in the smBrg1 KO mice, may be a result of pathological changes rather than a direct affect of Brg1 on miR-133 expression. However, we noted that there is a very large decrease in miR-133 expression levels in the primary colon SMCs as compared to intact tissue such that expression is barely detectable (Figure 15C). The very low levels of expression may thus obscure the additional effects of the Brg1 on miR-133 levels in the cultured colon SMCs.

iii. DN-Brg1 attenuates myocardin-mediated induction of miRs-143/145 and miR-133a.

To circumvent problems resulting from lowered microRNA expression in primary SMC cultures, we examined microRNA expression in a cell system in which smooth muscle differentiation is induced. Over-expression of myocardin in 10T1/2 cells has been previously shown to increase expression of most smooth muscle-specific genes and this increase can be blocked by DN-Brg1 [109,185]. Myocardin is a potent coactivator of SRF that is restricted to cardiac and smooth muscle tissues and is essential for vascular smooth muscle differentiation [186]. Myocardin has also been shown to induce expression of miR-143 in 10T1/2 cells [172]. We therefore used this system to examine the effects of DN-Brg1 on microRNAs. Similar to the smooth muscle-specific contractile proteins,

expression of miR-143, 145 and 133a were increased by myocardin and this induction was attenuated by DN-Brg1 (Figure 16). In contrast, myocardin did not induce miR-133b expression, implying that miR-133b may not be regulated by myocardin directly.

iv. Knockdown of myocardin or SRF in primary SMCs attenuates miRs-143/145 expression.

To further examine the role of SRF and myocardin in regulating expression of miRs-143/145 and 133 in smooth muscle cells, we knocked down myocardin or SRF in primary SMCs using shRNA. The qRT-PCR analysis confirmed knockdown of myocardin and SRF and subsequent attenuated expression of smooth muscle-specific contractile proteins (Figures 17A,B). As expected, miR-143 and 145 were reduced when either myocardin or SRF expression was knocked down (Figures 17A,B). In contrast, myocardin knockdown had no significant effect on miR-133a or 133b expression (Figure 17B), while SRF knockdown decreased expression of miR-133a and 133b (Figure 17A).

v. Brg1 together with myocardin/MRTFA synergistically induces expression of miRs-143/145, but not miRs-133a/b.

To further explore the role of Brg1 in regulating expression of microRNAs, we utilized SW13 cells lacking endogenous Brg1 or Brm expression. In these cells,

we found that myocardin or MRTFA's ability to induce expression of miRs-143/145 is dependent on Brg1 (Figure 18). SW13 cells were transduced with adenoviruses encoding Brg1, myocardin, MRTFA or YFP control. Western blots confirmed the successful expression of exogenous Brg1, myocardin or MRTFA in SW13 cells (Figure 18). Brg1, myocardin, or MRTFA alone did not dramatically influence the expression of any of the four microRNAs analyzed, whereas, Brg1 together with myocardin or MRTFA robustly induced the expression of miR-143 and miR-145 about 20 and 10-fold, respectively (Figure 18). Although myocardin alone up-regulated the expression of miR-133a about 2-fold this was not further enhanced by Brg1 (Figure 18). In contrast, miR-133b expression was not significantly affected by myocardin either in the presence or absence of Brg1. Together these data suggest that expression of miRs-143/145 is induced by myocardin or MRTFA in a Brg1-dependent manner, whereas miR-133a is only weakly activated by myocardin and miR-133b is refractory to myocardin and MRTFA activation. Similar results were obtained when Brm was used in place of Brg1 (data not shown).

vi. Myocardin increases SRF binding to the miRs-143/145 promoter in a Brg1 dependent manner.

Brg1 has been shown to enhance myocardin or MRTFA-mediated activation of smooth muscle-specific genes via facilitating SRF binding to their promoter regions [109,178]. As there is a potential SRF binding site in the promoter region

of miRs-143/5 locus, we thus determined if miRs-143/145 transcription was regulated in a similar manner. Binding of SRF to the promoter region of telokin was used as a positive control (Figure 19).

In SW13 cells, over-expression of myocardin in the absence of Brg1 caused only a very small increase in binding of SRF to the miRs-143/145 (Figure 19). However, in the presence of Brg1, myocardin caused a much greater increase in the binding of SRF to the promoter regions of miRs-143/145 (Figure 19). Meanwhile, myocardin Brg1 binding to the promoter region of miRs-143/145 was not significantly changed by Brg1. Neither was Brg1 binding altered by myocardin (Figure 19).

The ChIP data showed that Myocardin-mediated SRF binding to the miRs-133a promoter is also Brg1-dependent (Figure 19). However, the increase of SRF binding is not sufficient to drive the transcription of miR-133a, although it is part of the regulation mechanism. Using bioinformatic tools, we found several other important transcription factors' binding sites around the miR-133a loci, such as E-box element, and MEF2 binding sites (Figure 19). However, they were not able to increase miR-133 expression either.

vii. SRF binding to the regulatory regions of miRs is changed during SMC differentiation.

To further explore the SRF binding to the regulatory regions of miRs during differentiation, we utilized the myocardin-mediated SMC differentiation system from 10T1/2 cells. ChIP assays showed increases of SRF binding to the promoters of miRs-143/145 and miR-133a (Figure 20). Only Brg1 binding to the miRs-143/145 promoter was slightly enhanced during SMC differentiation.

We also analyzed the SRF binding to the regulatory regions of miRs in the colon smooth muscle tissues between control and smBrg1 knockout mice. Consistently, SRF binding was also reduced when Brg1 was knocked out in smooth muscle cells (Figure 20).

E. Discussion

Our current results demonstrate the SWI/SNF chromatin remodeling complex acts together with transcription factors and cofactors to regulate the expression of microRNAs in smooth muscle cells. SWI/SNF complex is required for myocardin or MRTFA to induce expression of miRs-143/145. These data, together with previous reports[109,178], suggest that Brg1 or Brm binding to myocardin or MRTFA recruits SWI/SNF to the miRs-143/5 promoter region, opening the chromatin structure thereby facilitating the tight binding of SRF to the promoter and subsequent transcriptional activation (Figure 21). Although Brg1 is also required for miR-133 expression *in vivo* and SRF is involved in the regulation of miR-133 expression, myocardin is a weak activator of miR-133a and does not significantly activate miR-133b, suggesting that Brg1 regulates miR-133 expression through other distinct mechanisms (Figure 21).

The Brg1 and myocardin/MRTFA dependent regulation of miRs-143/145 is very analogous to the previously reported mechanism by which these proteins regulate expression of many smooth muscle-specific contractile proteins [109,178]. These findings are also consistent with previous studies that have shown that miRs-143/145 expression can be regulated by myocardin and SRF. Myocardin has been shown to be able to increase miRs-143/145 expression in cardiomyocytes [167] and 10T1/2 cells [172] and a CArG box within the miRs-143/145 promoter has been shown to be required for reporter gene expression in

smooth muscle cells *in vivo* in mice [167,171]. The current studies extend these results to demonstrate that both myocardin and SRF are required for expression of miRs-143/145 in smooth muscle cells and that the SWI/SNF complex is required for myocardin-mediated induction of these microRNAs. These findings reveal that in smooth muscle cells the gene encoding miRs-143/145 is subjected to similar transcriptional and epigenetic regulation to genes encoding contractile proteins.

The decreased expression of miRs-133a/b *in vivo* in smBrg1 knockout mice is consistent with previous studies that showed that Brg1 plays a critical role in the regulation of miR-133 expression in skeletal muscle. In skeletal muscle cells, it has been proposed that MyoD recruits Brg1 to E box sequences within the miR-133a promoters [127]. As MyoD is restricted to skeletal muscle cells, clearly other transcription factors must recruit Brg1 to the miR-133 promoters in smooth muscle cells. Analysis of the regulatory regions of all three miR-133 genomic loci, revealed the presence of several highly conserved cis elements, including E box elements and potential binding sites for SRF and MEF2 (Figure 19). Knockdown of SRF in primary cultures of smooth muscle cells resulted in a small decrease in expression of both miR-133a and miR-133b suggesting that SRF may play a role in regulating expression of these genes in smooth muscle cells (Figure 17A). However, unlike miRs-143/145, expression of miR-133 genes was not significantly affected by myocardin knockdown (Figure 17B). In addition, myocardin was a very poor activator of miR-133a in SW13 cells and 10T1/2 cells

and miR-133b expression was completely refractory to myocardin stimulation in either cell type (Figure 17,18). The dramatic decrease in miR-133 expression seen in cultured smooth muscle cells (Figure 14C) suggests that results obtained from these cells should perhaps be interpreted with caution. The importance of SRF in regulating the expression of miR-133 thus remains to be determined *in vivo*. E-box binding proteins and MEF2, have been reported to play important roles in regulating expression of smooth muscle differentiation genes [187,188,189], however, the role of these proteins in regulating microRNA expression in smooth muscle has not been determined. As MEF2 has also been shown to bind to myocardin to autoregulate its own expression in cardiomyocytes [190] we evaluated the possibility that myocardin may cooperate with MEF2 proteins to induce miR-133 expression. However, in the SW13 cell system MEF2 proteins with or without myocardin were not able to induce miR133 expression even in the presence of Brg1. Similarly, E12/47 proteins with Brg1 were not able to induce miR-133 expression either. Additional studies will thus be required to determine the mechanism regulating the expression of miR-133 in smooth muscle cells.

SWI/SNF complexes contain one of two alternative ATPases, Brahma-related gene 1 (Brg1) or Brahma (Brm). Both of these proteins are ubiquitously expressed in almost all tissues, and have both redundant and distinct functions in regulating gene expression. *In vitro*, both Brg1 and Brm can facilitate myocardin or MRTFA-mediated induction of smooth muscle-specific genes [109,178] and

miRs-143/145 (Figure 18 and data not shown). However, the knockout of Brg1 alone in smooth muscle cells, *in vivo*, resulted in decreased expression of smooth muscle-specific contractile proteins and microRNAs in the gastrointestinal tract [182] (Figure 14). Thus, *in vivo*, Brg1 must have specific functions in smooth muscle cells that cannot be performed by Brm. Why there are specific requirements for Brg1 *in vivo* but not *in vitro* remains a mystery but may reflect expression levels of the proteins *in vivo*. *In vitro*, in over-expression experiments it is possible that Brm may be able to access complexes which Brg1 cannot access at normal expression levels *in vivo*. Specific functions of Brg1 have also been revealed in other cell types. For example, Brg1 is specifically required for T-cell development [191]. Moreover, global Brg1 null mice die early in embryonic development due to growth arrest of the inner cell mass and trophoblast [192], while global Brm-null mice are viable and slightly larger than normal [193]. Knockout of Brg1 in smooth muscle cells in mice results in a myriad of defects including altered vascular remodeling leading to persistent ductus arteriosus and altered gastrointestinal contractility leading to intestinal blockage [182]. In addition to the described decreased expression of miRs-143/5 and miR-133 in smBrg1 knockout mice, we also observed significantly increased levels of several microRNAs. This observation suggests that either these microRNAs are transcriptionally repressed by Brg1, or they are increased as a result of pathological changes that occur in the knockout mice. As DN-Brg1 did not increase expression of these microRNAs in smooth muscle cells *in vitro* (data not shown), it is probable that the changes observed *in vivo* are not a direct result of

Brg1-mediated repression. It is likely that the combined effects of altered expression of protein coding genes and microRNAs contribute to the complex pathology observed in the smBrg1 knockout mice. In support of this, miRs-143/145 knockout mice have been shown to exhibit decreased vascular tone and decreased differentiation [167,169] suggesting that decreased expression of these microRNAs may be contributing to the impaired contractility observed in the smBrg1 knockout mice.

In conclusion, we demonstrated for the first time that the SWI/SNF chromatin remodeling complex plays an important role in regulating microRNA expression in smooth muscle cells, *in vitro* and *in vivo*. Transcription of miRs-143/145 is regulated by SRF/myocardin complex, in a SWI/SNF chromatin remodeling complex-dependent manner. In contrast, expression of miR-133 genes appears to be largely myocardin independent. In smooth muscle cells, microRNAs and protein coding genes can thus be regulated by similar transcriptional and epigenetic mechanisms.

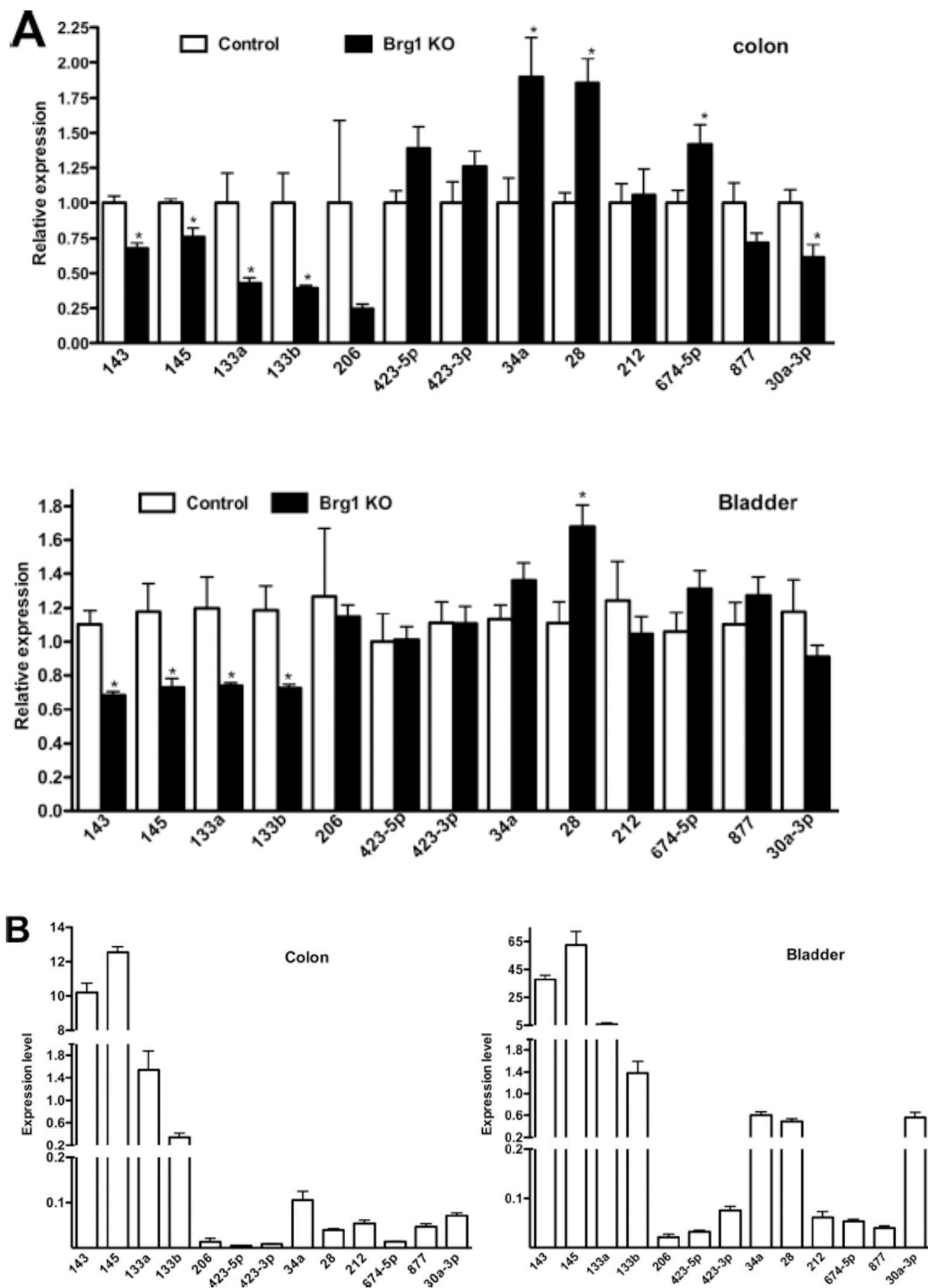


Figure 14. MicroRNA differences between control and Brg1 knockout mice

A. Total RNAs were extracted from colon (n=7) and bladder (n=6) of both at 6-8 weeks old control (open bars) and Brg1 knockout mice (filled bars). The cDNA were generated and microRNA expression quantitated by real-time qPCR. MicroRNA expression was normalized to expression of U6 RNA as an internal control and is expressed relative to levels in control mice. Relative expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{smBrg1KO} - Ct_{U6}) - (Ct_{control} - Ct_{U6})$. *, p<0.05.

B. Relative abundance of microRNAs in colon and bladder. Expression levels of miRs in colon and bladder from control mice are shown normalized to U6 RNA expression (n=6-7). *, p<0.05. Error bars, standard deviation.

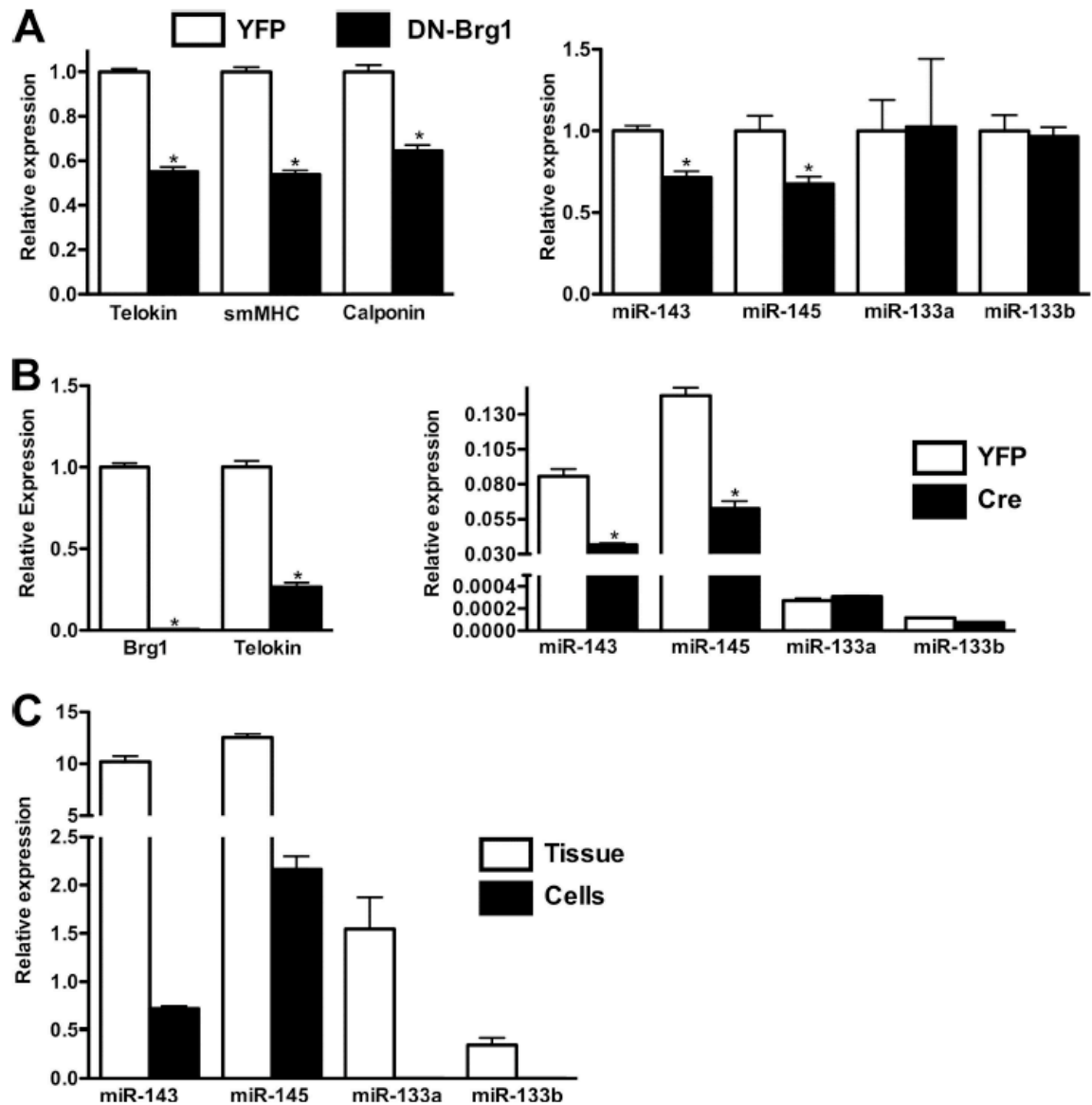


Figure 15. Brg1-dependent changes in mRNAs and microRNAs in primary smooth muscle cells.

A. Primary smooth muscle cells were collected from colon smooth muscle tissues of wild type mice and were transduced by adenovirus expressing YFP (open bars) as control (n=6) or DN-Brg1 (closed bars, n=5). Two days later, cells were lysed and total RNA isolated. The qRT-PCR experiments were performed to

compare the expression of smooth muscle markers and microRNAs. The mRNA and microRNA expression were normalized to HPRT and U6 internal controls, respectively, and then expressed relative to YFP control samples. Relative expression = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t_{DNBrg1}} - C_{t_{HPRT/U6}}) - (C_{t_{YFP}} - C_{t_{HPRT/U6}})$.

B. Primary smooth muscle cells were collected from colon smooth muscle tissues of Brg1^{flox/flox} mice and were transduced by adenovirus expressing YFP (open bars) as control or Cre (filled bars) (n = 6). The mRNA and microRNA expression levels were quantitated as described in A.

C. Comparison of microRNA expression levels in colonic smooth muscle tissue (open bars) and primary cultures of colonic smooth muscle cells is shown (filled bars). MicroRNA expression was normalized to expression of U6 internal control.

*, p < 0.05. Error bars, standard deviation.

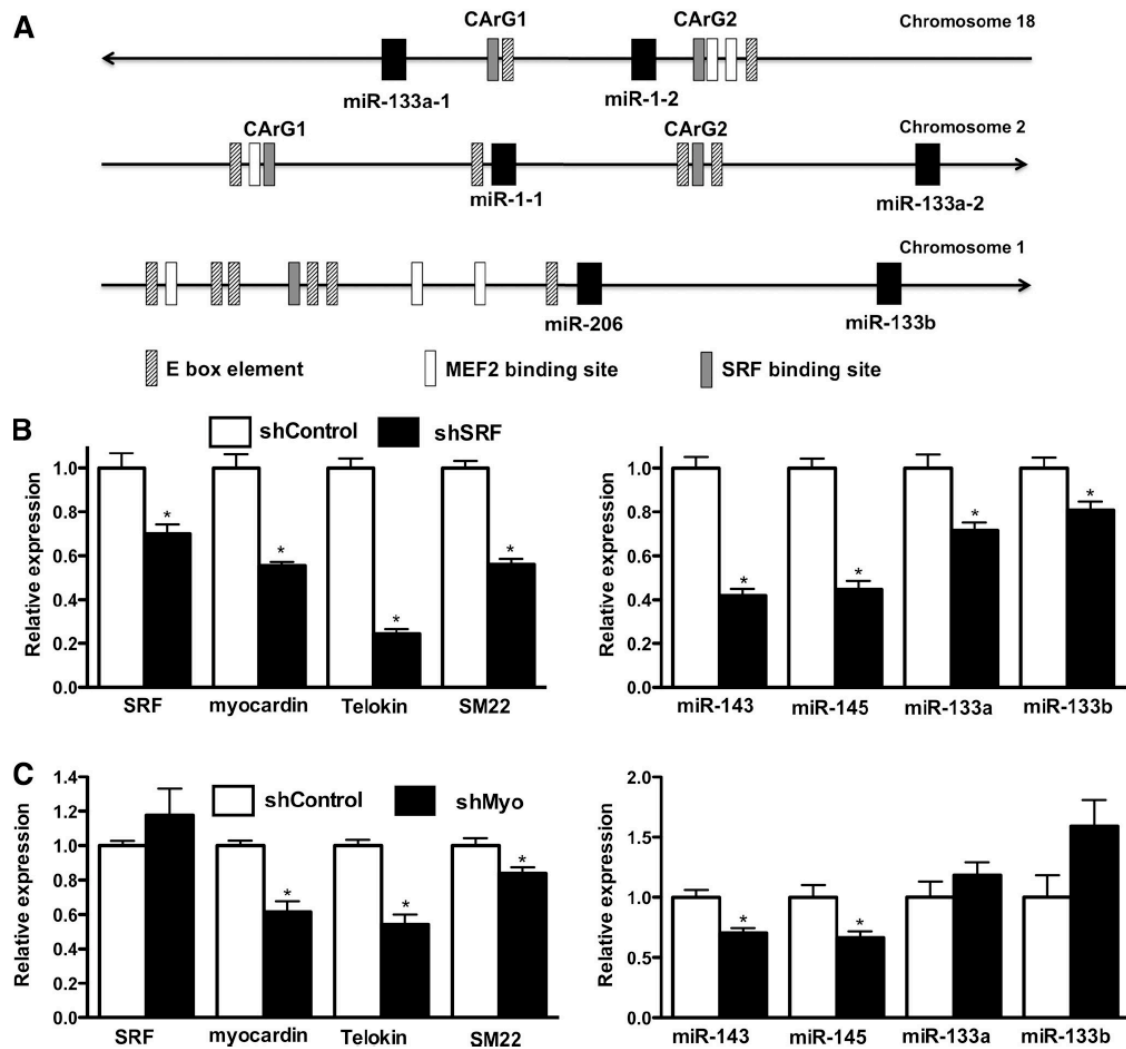


Figure 16. Knockdown of myocardin or SRF in primary smooth muscle cells affects microRNA expression.

A. Schematic representation of miR-133a and 133b loci. Conserved potential regulatory regions are indicated.

B and C. primary colonic smooth muscle cells seeded in 6-well plates. Endogenous SRF (B) or myocardin (C) was knocked down by transduction with adenovirus encoding shSRF or shMyocardin, respectively (solid bars).

A non-targeting shControl virus was used as control (open bars) (n=4-8). 48 h

later, total RNA was harvested. The mRNA and microRNA levels were quantitated by qRT-PCR as described in the previous figure and they were expressed relative to levels in shControl virus transduced samples.

*, $p < 0.05$. Error bars, standard deviation.

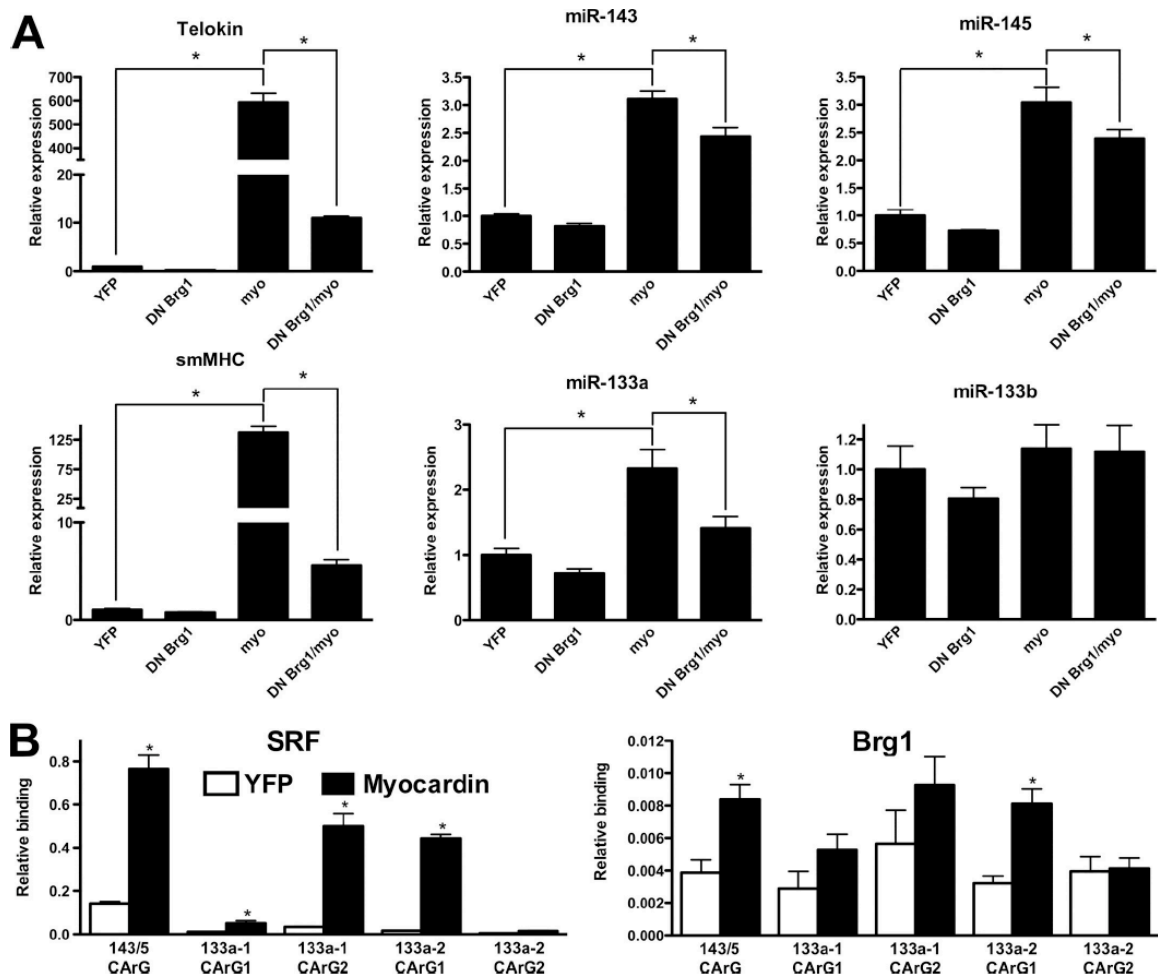


Figure 17. Brg1-dependent changes in mRNA and microRNA expression during myocardin-mediated induction of smooth muscle cell differentiation in 10T1/2 cells.

A, 10T1/2 cells were seeded in 6-well plates and transduced with YFP adenovirus or DN-Brg1 adenovirus, with or without myocardin (myo) adenovirus as indicated. 48 h later total RNA was harvested for qRT-PCR analysis to detect the expression levels of smooth muscle-specific genes and microRNAs as described previously, 10T1/2 cells were transduced with YFP (open bars) or myocardin (filled bars) adenovirus. Two days later, chromatin was harvested and

analyzed by ChIP assays for the binding of SRF and Brg1 to the CArG boxes indicated previously (n=4). ChIP samples were analyzed by real-time qPCR. Signals from immunoprecipitated samples were normalized to corresponding input samples and then presented relative to the signal obtained from the samples transduced with YFP adenovirus. Relative expression= $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{myo} - Ct_{input}) - (Ct_{YFP} - Ct_{input})$. *, p < 0.05. Error bars, standard deviation.

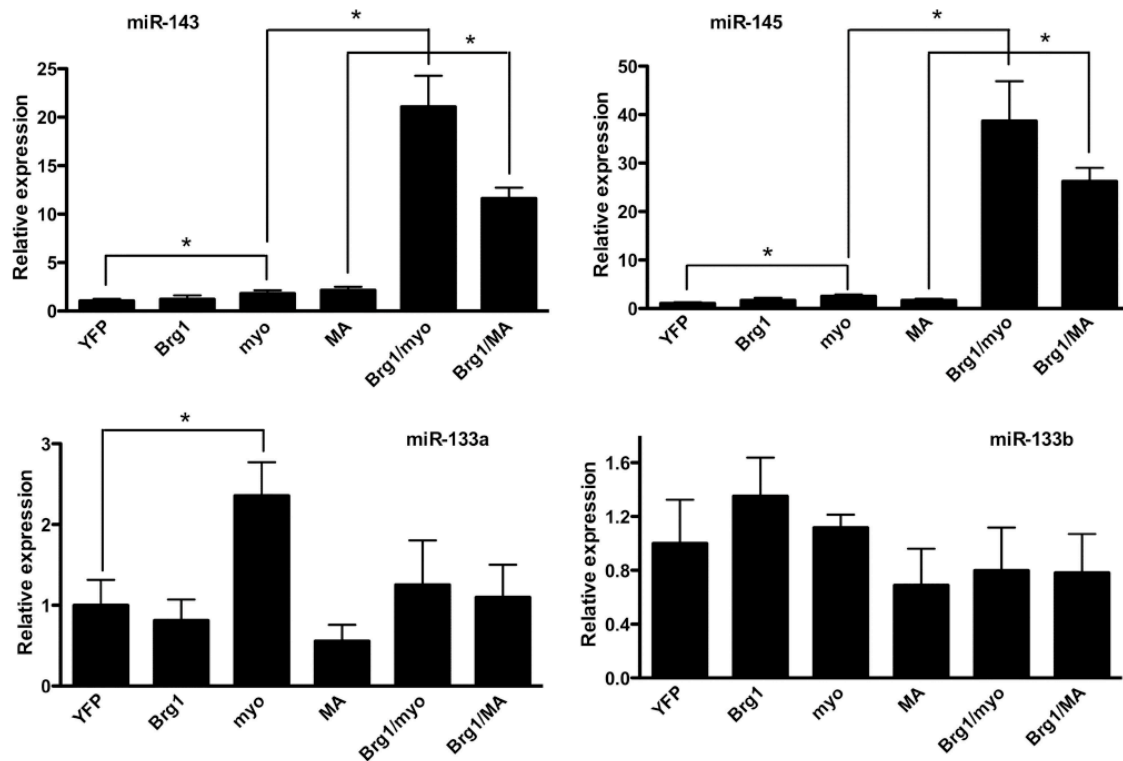


Figure 18. Brg1 and myocardin or MRTFA synergistically induce miRs-143/145, but not miRs-133a/133b in SW13 cells.

SW13 cells were transduced with YFP, Brg1, myocardin (myo), or MRTFA (MA) adenovirus alone or in combination, as indicated. 48 h after transduction total RNAs were harvested for qRT-PCR analysis as described previously (n = 6-8).

*, p < 0.05. Error bars, standard deviation.

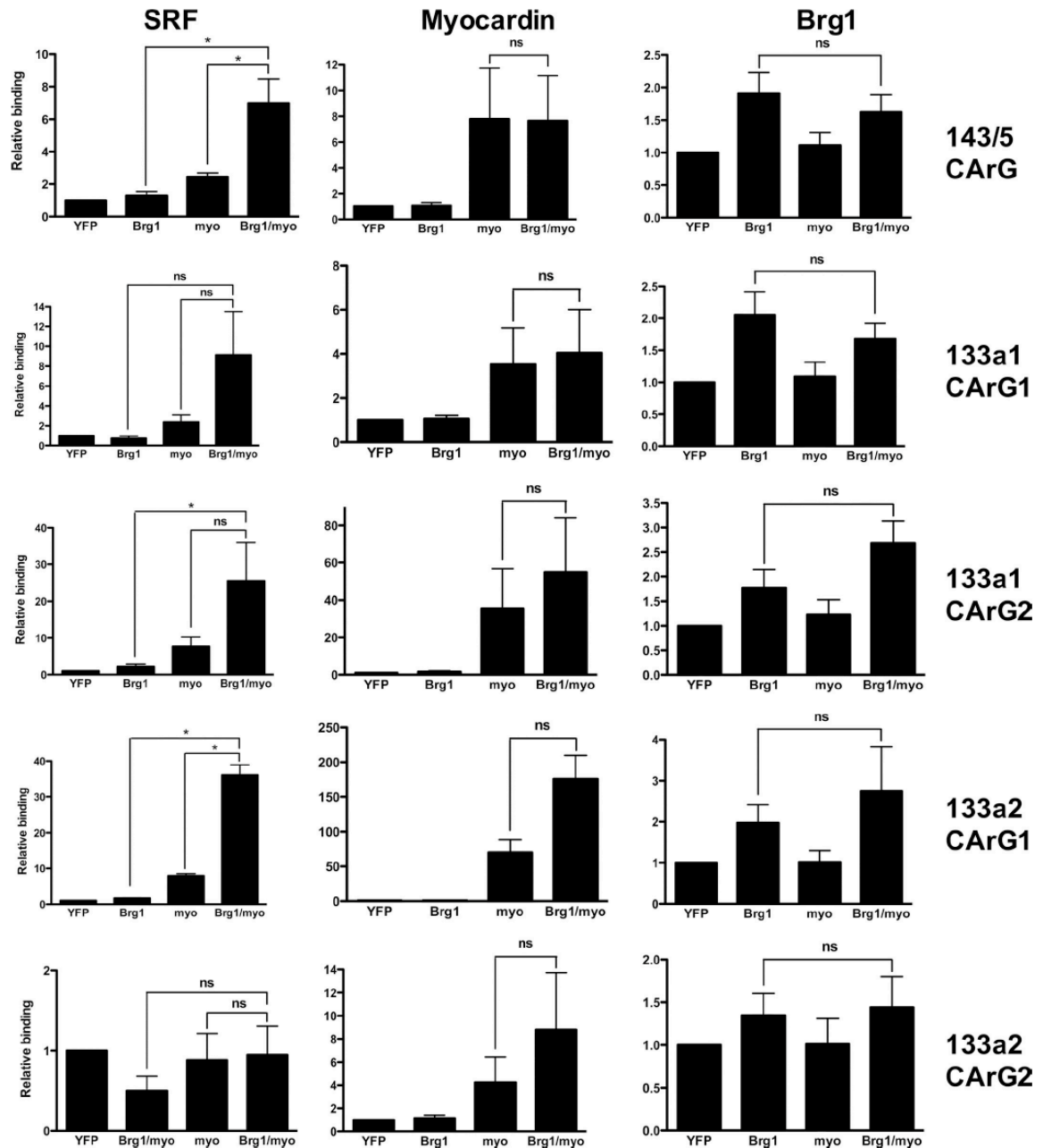


Figure 19. ChIP analysis of SRF, myocardin, and Brg1 binding to the CArG elements of the miR-143, 145, and 133a genes in SW13 cells.

SW13 cells were transduced with adenovirus encoding YFP, Brg1, or myocardin (myo), as indicated. 48 h later, chromatin was harvested and analyzed by ChIP

assays using either an SRF, omni (to detect the epitope tag on myocardin), or Brg1 antibody or IgG control (n = 5–7). Specific primers were utilized to detect the CArG elements in the promoter regions of the telokin gene (data not shown), miRs-143/145 and miR-133a loci by quantitative PCR. Signals from immunoprecipitated samples were normalized to corresponding input samples and are then presented relative to the signal obtained from the samples transduced with YFP adenovirus as described previously.

*, $p < 0.05$; ns, not significant ($p > 0.05$). Error bars, standard deviation.

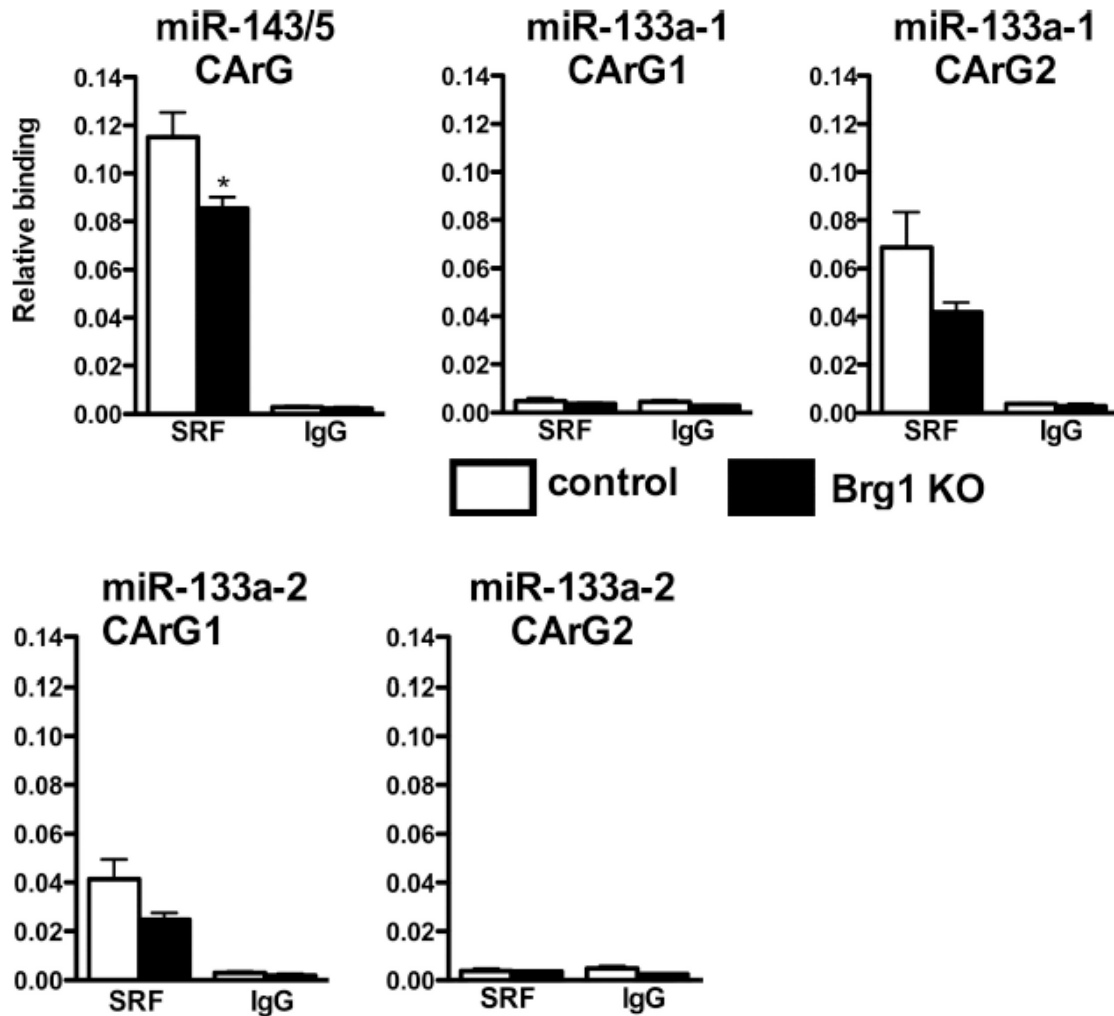


Figure 20. ChIP analysis of SRF binding to the miR-143, 145, and 133a loci in colonic smooth muscle tissues from smBrg1 knockout mice.

Colonic smooth muscle tissues were collected from control (open bars) and smBrg1 KO (filled bars) mice, and chromatin was harvested for ChIP assays (n = 6). Chromatin was immunoprecipitated with an SRF antibody or IgG control. CARG elements within the miRs-143/145 and miR-133a loci were detected by qPCR. The qPCR signals from immunoprecipitated samples were normalized to

corresponding input samples. Relative expression= $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{control/ko-}}$
 Ct_{input} . *, $p < 0.05$. Error bars, standard deviation.

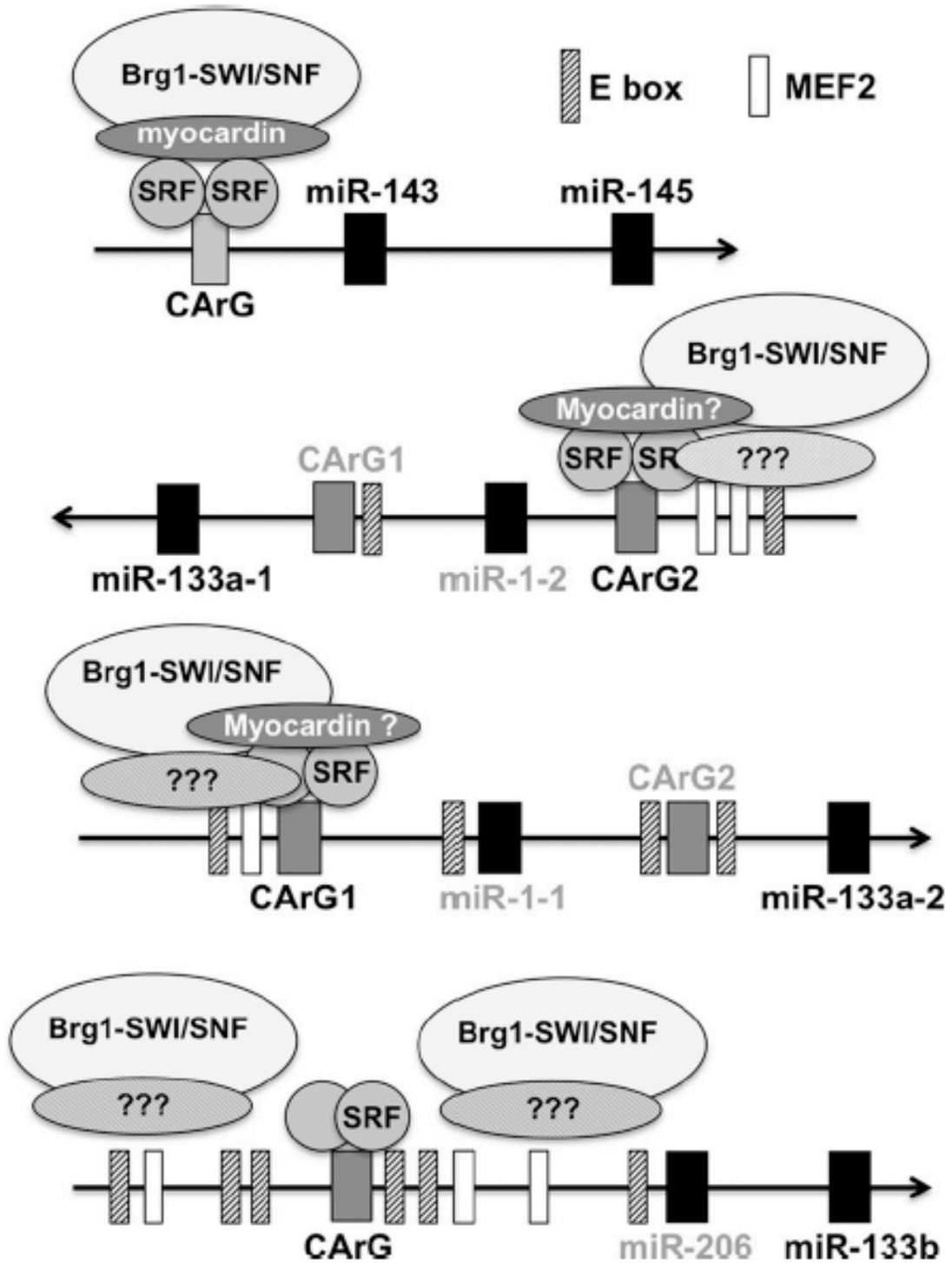


Figure 21. Regulation of miRs-143/145 and miRs-133a/b by SRF and Brg1

Schematic models (not to scale) show the mechanisms by which Brg1 regulates

miRs-143/145, miR-133a-1, miR-133a-2, and miR-133b loci. Myocardin promotes SRF binding to a single CArG box in the miRs-143/145 promoter in a Brg1-dependent manner that likely involves direct binding of myocardin to Brg1. This is sufficient to drive transcription of miRs-143/145 in smooth muscle cells. Only one of the two CArG boxes within each of the miR-133a loci binds to SRF in smooth muscle cells. Although myocardin can stimulate SRF binding to these CArG boxes in a Brg1-dependent manner this is not sufficient to stimulate miR-133a transcription. Other transcription factors must act in concert with SRF/myocardin to drive miR-133a expression in smooth muscle cells. Expression of miR-133b is independent of myocardin but dependent on SRF. As we have previously shown that SRF does not bind to Brg1, Brg1 must regulate miR-133b expression through interactions with other transcription factors. Black boxes, miR stem loop structures; solid gray boxes, CArG elements; hatched boxes, E box elements; open boxes, potential MEF2 binding sites.

ChIP Primers	
human telokin ChIP F	GTC AGC AAT AAG TCT TTG GAG CTG TCT CAG
human telokin ChIP R	ATG GCT CTC ACA GCA TTG CCC GTT TTC TGG
mouse miR-143/145 CArG ChIP F	CTAGCTCAGAGCAGCCTTG
mouse miR-143/145 CArG ChIP R	CCCGGGAGGGGTGAGAG
human miR-143/145 CArG ChIP F	AGCAAACCTCTAACACTGTG
human miR-143/145 CArG ChIP R	GCCTTGCAAGCCACACTATC
mouse miR-133a-1 CArG1 ChIP F	TGTACTGGCCATATCAGGACAA
mouse miR-133a-1 CArG1 ChIP R	AACACTTCAACTGTCACCCTGA
human miR-133a-1 CArG1 ChIP F	CATATCGGGGCAAGGAGAG
human miR-133a-1 CArG1 ChIP R	CCTGACAGGGATAACAGATGC
mouse miR-133a-1 CArG2 ChIP F	TCTCTTGTGCCTCTCTCTTCC
mouse miR-133a-1 CArG2 ChIP R	CGACAATCGACAGCTGAGAG
human miR-133a-1 CArG2 ChIP F	GTGCCTCTCTCTTCCCTGAC
human miR-133a-1 CArG2 ChIP R	CTTCCTCAGCTTTTGGATCG
mouse miR-133a-2 CArG1 ChIP F	GCCCAGCTGACATGCTATTT
mouse miR-133a-2 CArG1 ChIP R	ATATACCCCGCCAAACATCC
human miR-133a-2 CArG1 ChIP F	CCCAGCTGACAGGCTATTTT
human miR-133a-2 CArG1 ChIP R	GCCAAGCATCCCAAACAC

mouse miR-133a-2 CArG2 ChIP F	CCCATGAGCCCTCAATCAT
mouse miR-133a-2 CArG2 ChIP R	ATGACATGGGCAAGAGTGG
human miR-133a-2 CArG2 ChIP F	CCCTCTGTCATGCCCAGATA
human miR-133a-2 CArG2 ChIP R	GCTGAGAACGCGGACTATTT

Table 1. Primers used for ChIP assay

CHAPTER IV

CONCLUSIONS AND FUTURE STUDIES

A. CArG-dependent regulation of smMLCK

Results of my first project clearly show that a CArG box-containing region in intron 15 of the *mylk1* gene is required for expression of the 130kDa smMLCK. Deletion of this intronic CArG box attenuates expression of the 130kDa smMLCK without affecting expression of either the 220kDa MLCK or telokin. Moreover, decreased expression of only the 130kDa smMLCK in smooth muscle tissues attenuates smooth muscle contractility associated with decreased myosin light chain phosphorylation and impairs small intestine smooth muscle proliferation.

To extend these studies it would be interesting to determine if the altered intestinal contractility measured *ex vivo* translates to impaired intestinal motility *in vivo*. To achieve this, control mice and the knockout littermates would be orally administered contrast agent Gastrografin, then anesthetized for Computed Tomography (CT) to monitor the progress of the contrast agent through the GI tract over time. From this measurement I would be able to calculate GI transit time to determine if the decreased expression of the smMLCK impairs motility.

Similarly, to further evaluate the physiological effects of the observed decreased contractility of isolated aortic segments, it would be important to measure the basal and agonist-stimulated changes in blood pressure of both control and knockout mice *in vivo*. To do this I would use telemetry methods to record their blood pressure *in vivo*. The basal blood pressure as well as the agonist-stimulated blood pressure (e.g. Angiotensin II) would be recorded continuously for several days to determine if the decreased expression of the smMLCK in vascular SMCs affects blood pressure.

These additional studies together with my current findings would solidify the importance of the 130kDa smMLCK isoform in regulating smooth muscle contractility *in vivo*. This then raises the question of what are the functions of the 220kDa MLCK in smooth muscle cells. Through specific targeting of a portion of the catalytic domain shared by the 220 and 130kDa MLCKs, it has been reported that ablation of both MLCK isoforms in smooth muscle cells resulted in impaired contractility and decreased myosin light chain phosphorylation [8,150]. Based on our data demonstrated in Chapter II, I think it is the 130kDa smMLCK that plays an important role in mediating smooth muscle contraction. However, to confirm that, I would generate smooth muscle-specific 220kDa MLCK null mice using a similar approach to the 130kDa intronic knockout mice and analyze smooth muscle contractility in control and 220kDa MLCK knockout mice as described above.

In my study, I only carried out the research in SMCs. However, the intronic CArG floxed mouse we generated would be a great tool for interrogating the specific function of the 130kDa MLCK in other cell types, such as endothelial cells. In this case, Tie 2-Cre expressing mice would be mated with the intronic CArG floxed mice to generate endothelial cell specific 130kDa MLCK knockdown. With only the 130kDa MLCK isoform expression altered, it will be possible to determine the specific role of 130kDa MLCK in regulating endothelial cell functions.

As I mentioned in Chapter II, a Notch responsive element has been identified at -3687bp upstream of transcription start site and plays an important role in activating 130kDa smMLCK expression in vascular smooth muscle cells [162]. However, this element is not included in the reporter gene described in my study, which extends from -389 to +8427. This may explain the reason why our reporter genes exhibited very low levels, or undetectable LacZ expression in vascular smooth muscle tissues. To confirm this, I would utilize a reporter gene, containing the region from -3687 Notch responsive element to +8427, to generate the reporter mice. Analysis of the reporter mice in a similar manner to that in my study would demonstrate the role of the upstream Notch responsive element in directing gene expression in vascular SMCs.

B. Transcriptional and epigenetic regulation of microRNAs in SMC

Data described in Chapter III demonstrate the SWI/SNF chromatin remodeling complex acts together with transcription factors and cofactors to regulate the expression of microRNAs in smooth muscle cells. Specifically, SWI/SNF complex is required for myocardin or MRTFA to induce expression of miRs-143/145, so that it further opens the chromatin structure thereby facilitating the tight binding of SRF to the promoter and subsequent transcriptional activation. Although Brg1 is also required for miR-133 expression *in vivo* and SRF is involved in the regulation of miR-133 expression, myocardin is a weak activator of miR-133a and does not significantly activate miR-133b, implying that Brg1 regulates miR-133 expression through other distinct mechanisms.

Although these studies have provided new information on the regulation of microRNA expression in smooth muscle cells, a number of key questions remain to be answered as detailed below.

i. What other transcription factors regulate transcription of miR-133 in VSMCs?

Although Brg1 is also required for miR-133 expression *in vivo* and SRF is involved in the regulation of miR-133 expression, myocardin does not significantly activate miR-133b, implying that Brg1 must regulate miR-133 expression through other distinct transcription cofactors rather than myocardin.

In addition to SRF, highly conserved E-box elements and MEF2 binding sites are present in the regulatory region of miR-133.

To determine if these sites are important for regulating miR-133 expression, I would first determine whether these transcription factors are able to bind to the cis-acting elements within SMC by chromatin immunoprecipitation (ChIP) assays using antibodies specific for transcription factors MEF2, and E-box binding proteins E12, HEB, E2-2, E47, USF1, USF2. Once any transcription factor is proved to be able to bind to regulatory region, the importance of the predicted transcription factor binding sites for the transcription of miR-133 would be tested by luciferase assays. To do this, luciferase reporter genes containing mutations in each of the identified cis-acting elements would be generated and transfected into SMCs and luciferase activity measured. Finally to determine if specific transcription factors are important for the expression of miR-133, I would knockdown expression of these proteins in primary SMCs and examine the subsequent changes in miR-133 expression by qRT-PCR.

ii. How does Brg1 act together with the identified transcription factors to regulate expression of miR-133 in SMCs?

Once potential transcription cofactors are identified, I would thus determine if the transcription factors serve to recruit Brg1 to the miR-133 gene by direct interaction between the transcription factors and Brg1. This would be revealed by

co-immunoprecipitation assays and chromatin immunoprecipitation assays. The former experiments would be able to identify whether Brg1 physically interacts with the identified transcription factors. For the latter experiments, I would knockout the transcription factors and utilize the specific antibody against Brg1 to pull down the protein complex together with bound DNA fragments. I would perform PCR to amplify the targeted regulatory element using designed primers flanking the binding sites and then the amount of Brg1 bound to the regulatory region with or without the identified transcription factors would be compared.

Once I have determined how chromatin remodeling complexes are recruited to the microRNA genes I would then confirm the functional importance of these interactions using the SW13 cell system described in Chapter III. In this experiment, Brg1 and/or transcription factor candidates would be over-expressed in SW13 cells. RNA would be extracted and expression of miR-133 would be determined by real-time PCR. This approach will allow me to determine if specific transcription factors can induce miR-133 expression in the presence or absence of Brg1.

After I have established how expression of miR-133 is regulated using these *in vitro* systems the next step would be to test this *in vivo*. Ideally, to do this the identified specific transcription factor would be knocked out from smooth muscle cells in mice and the resultant affects on miR-133 expression would be determined.

Together, my data suggest that similar to SMC contractile protein genes, there exists fine regulation of microRNA expression in SMCs. MicroRNAs have constituted a new layer of gene regulation for fine tuning the phenotype of smooth muscle cells. As miR-133 has been linked to SMC phenotypic switch during vascular diseases, unraveling the mechanisms controlling its expression will provide a better understanding of the mechanisms causing atherosclerosis and vascular restenosis. One could envisage the development of novel therapies targeting specific transcription cofactors that control expression of microRNAs to selectively fine-tune smooth muscle-specific gene expression and ameliorate or reverse pathological changes in smooth muscle cells that occur in many diseases.

C. Overall summary

In summary, my research demonstrates that through direct interaction with a CArG box in the *mylk1* gene, SRF is important for regulating expression of smMLCK to control smooth muscle contractility. In addition, SRF is able to harnesses epigenetic mechanisms to modulate expression of smooth muscle contractile protein genes directly and indirectly via changes in microRNA expression. Together these mechanisms permit SRF to coordinate the complex phenotypic changes that occur in smooth muscle cells. Furthermore, the I_{CArG} flox mice provide a novel model system for further interrogating the specific functions

of the 130kDa smMLCK isoform in different cell types *in vivo*. Also, unraveling the genetic and epigenetic mechanisms that regulate microRNA expression in smooth muscle cells will help to fill the existing gap in our knowledge and broaden our understanding of the importance of non-coding RNAs in SMC pathophysiology.

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Peking University, Beijing, China (2001-2006)

Bachelor Degree of Medicine in Nursing

Professional Experience

- **Indiana University School of Medicine, Indianapolis, USA (2008-2013)**
 - Research Projects:
 - Epigenetic regulation and function of microRNAs in smooth muscle
 - Transcriptional regulation and function of smooth muscle myosin light chain kinase
 - Techniques: generation and analysis of knockout and transgenic mouse models, cell culture, FACS, IHC/HE staining, imaging (confocal, FRET), qRT-PCR, in situ hybridization, western blot, northern blot, molecular cloning, basic molecular biology techniques, ChIP, promoter/enhancer analysis, microarray analysis
- **Institute of Molecular Medicine, Peking University, Beijing, China (2006-2008)**
 - Research Assistant, with graduate-level training in biochemistry and molecular biology
 - Research projects:
Mechanism and function of components of Traditional Chinese Herbs (Salvia miltiorrhiza and Sanqi) in cardiovascular diseases
- **People's Hospital, Peking University, Beijing, China (2004-2006)**
 - Intern Student, with clinical rotation in the departments of Internal Medicine, Surgery, Gynecology, Obstetric, Pediatrics, Emergency Room and Operating Room
 - Learnt basic biomedical science, and the etiology, diagnosis, treatment, nursing and prevention of diseases

Publications

- **Chen M**, Zhang W, Lu X, Gunst SJ, Kassab GS, Tune JD, Herring BP. Regulation of smooth muscle myosin light chain kinase expression by an intronic CArG element. (Under review in JBC)
- **Chen M**, Herring BP. Regulation of microRNAs by Brahma-related gene 1 (Brg1) in smooth muscle cells. *Journal of Biological Chemistry*. 2013, 288(9): 6397-6408
- Zhang M, **Chen M**, Kim JR, Zhou J, Jones RE, Tune JD, Kassab GS, Metzger D, Ahlfeld S, Conway SJ, Herring BP. Brahma and Brahma related gene 1 containing SWI/SNF complexes play distinct roles in gastrointestinal smooth muscle development. *Molecular and Cellular Biology*. 2011, 31(13): 2618-2631
- Rodenberg JM, Hoggatt AM, **Chen M**, Touw K, Jones RE, Herring BP. Regulation of Serum Response Factor activity and smooth muscle cell apoptosis by Chromodomain Helicase DNA-Binding Protein 8. *American Journal of Physiology-Cell Physiology*. 2010, 299(5): C1058-C1067
- Pan C, Huo Y, An X, Singh G, **Chen M**, Yang Z, Pu J, Li J. Panax notoginseng and its components decreased hypertension via stimulation of endothelial-dependent vessel dilatation. *Vascular Pharmacology*. 2012, 56(3-4): 150–158
- Pan C, Lou L, Huo Y, Singh G, **Chen M**, Zhang D, Wu A, Zhao M, Wang S, Li J. Salvianolic acid B and tanshinone IIA attenuate myocardial ischemia injury in mice by NO production through multiple pathways. *Therapeutic Advances in Cardiovascular Disease*. 2011, 5(2): 99-111
- **Chen M**, Sun Y, Wang L. The correlation between Sense Of Coherence (SOC) and Anxiety among hospitalized patients with coronary diseases. *Chinese Journal of Modern Nursing*. 2008, 14: 1572-1574
- **Chen M**, Zhang H. Advancement in research on Kawasaki Disease. *Foreign Medical Sciences: Section of Maternal and Child Health*. 2005,16(1): 34-36

Abstracts

- **Meng Chen**, Paul Herring. Regulation of smooth muscle myosin light chain kinase expression by an intronic CArG element. International MADS Box conference. 2013, Rochester, NY
- **Meng Chen**, Paul Herring. The role of Brahma-related gene 1 in regulating the expression of microRNAs in smooth muscle cells. Experimental Biology, joint with ASBMB annual meeting. 2013, Boston, MA
- **Meng Chen**, Paul Herring. Altered expression of Brahma-related gene 1-dependent microRNAs contributes to colonic dysmotility. Keystone Symposia: Non-coding RNAs, joint with meeting on Eukaryotic Transcription. 2012, Snowbird, UT

- **Meng Chen**, Paul Herring. The role of Brahma-related gene 1 in regulating the expression of microRNAs in colonic smooth muscle cells. Experimental Biology, joint with ASBMB annual meeting. 2012, San Diego, CA
- **Meng Chen**, Paul Herring. The role of Brahma-related gene 1 in regulating the expression of microRNAs in smooth muscle cells. Indiana Physiological Society annual meeting. 2012, Indianapolis, IN
- **Meng Chen**, Paul Herring. The role of 130kDa MLCK in regulating *in vivo* vascular permeability. Indiana Physiological Society annual meeting. 2011, Indianapolis, IN

Honors & Fellowship

- **American Heart Association (AHA) Pre-Doctoral Fellowship** (2012-2014)
- **International MADS Box Conference Travel Award** (2013)
- **American Society for Biochemistry and Molecular Biology (ASBMB) Graduate or Postdoctoral Travel Award** (2013)
- **Indiana University School of Medicine (IUSM) Travel Award** (2012)
- **ASBMB Graduate or Postdoctoral Travel Award** (2012)
- **Educational Enhancement Grant** for graduate student in IUPUI (2012)
- **University fellowship** for graduate student from Indiana University (2008-2009)
- **May Fourth Scholarship** of Peking University (PKU) (2007)
- **Outstanding Graduate** of the City of Beijing (2006)
- **Outstanding Graduate** of PKU (2006)
- **Elsevier Medical Scholarship** for Excellent Medical Student of PKU (2005/2003)
- **Second-prize Scholarship** for Excellent Medical Student of PKU (2004)
- **Top-prize Scholarship** for Excellent Medical Student of PKU (2002)
- **Merit Student in Academy, Morality and Health** of PKU (2005/2004/2003/2002)
- **Excellent Student Leadership Award** of Shandong Province of China (2001)

Professional Affiliations

- American Society of Biochemistry and Molecular Biology (2011-present)
- American Heart Association (2011-present)
- American Physiological Society (2010-present)

Teaching Training

- Preparing for Future Faculty Program (PFF)
- Class Facilitator for Course X604-Clinical Problem Solving (2012)